FUNCTION OF CUTICULAR WAXES IN PLANT RESPONSE TO WOUNDING

Dissertation

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Milena Lewandowska

born in Gdańsk, Poland

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Department of Plant Biochemistry

Albrecht-von-Haller-Institute for Plant Sciences

University of Goettingen

Thesis Committee:

Pro. Dr. Ivo Feussner, Department of Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Sciences, University of Goettingen

Prof. Dr. Andrea Polle, Department for Forest Botany and Tree Physiology, Buesgen-Institute, University of Goettingen

Prof. Dr. Ljerka Kunst, Department of Botany, University of British Columbia, Vancouver, Canada

Members of examination board:

Referee: Prof. Dr. Ivo Feussner, Department of Plant Biochemistry, Albrecht-von-Haller-Institute for Plant Science, University of Goettingen

2nd Referee: Prof. Dr. Andrea Polle, Department for Forest Botany and Tree Physiology, Buesgen-Institute, University of Goettingen

Further members of the Examination Board

Prof. Dr. Ljerka Kunst, Department of Botany, University of British Columbia, Vancouver, Canada

Prof. Dr. Christiane Gatz, Department of Plant Molecular Biology and Physiology, Albrecht-von-Haller-Institute for Plant Science, University of Goettingen

Prof. Dr. Volker Lipka, Department of Plant Cell Biology, Albrecht-von-Haller Institute for Plant Sciences, University of Goettingen

PD Dr. Marcel Wiermer, RG Molecular Biology of Plant-Microbe Interactions, Albrecht-von-Haller-Institute for Plant Sciences, University of Goettingen

Date of oral examination: 24th of June 2019

Table of contents

Affidavit

Hereby, I confirm that the dissertation *Function of cuticular waxes in plant response to wounding* has been written independently and no other sources and aids were used than quoted.

Milena Lewandowska,
Goettingen, May 2019

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Abstract

Neutral lipids, such as triacylglycerol (TAG) and wax are essential metabolites for plants. TAG is the most abundant storage compound in all eukaryotes. It plays an important role in plant germination but also in lipid homeostasis upon stress. Wax is a mixture of aliphatic compounds, which all together form a hydrophobic film, covering all aerial parts of plants. This lipid-based barrier protects plants from environmental threats and an uncontrolled loss of water. The biosynthesis of those two lipid classes is well studied, however little is known about its regulation upon stress. Hence, this thesis focused on revealing the function of wax and TAG biosynthesis upon wounding in *Arabidopsis thaliana*.

Wounding mimics herbivore attack or mechanical tissue disruption and is one of the most common plant stresses. The main wound-signal is the hormone jasmonoyl-isoleucine (JA-Ile), whereas wax and TAG biosynthesis was described to be regulated by on another hormone - abscisic acid (ABA). It is shown in this study that genes coding for enzymes of the surface wax synthesis as well as those of the last steps of the TAG biosynthesis are induced upon wounding, mainly in a JA-Ile-independent but ABA dependent manner. However, on the metabolite level, wax accumulation after wounding is diminished in JA-Ile-deficient plants and decreased in ABA-reduced plants. However, the enrichment in TAG is independent of JA-Ile and ABA signaling. To dissect the wound from the drought stress response, wounding experiments in high humidity were conducted. It was shown, that high humidity prevents the wound-induced wax accumulation in Arabidopsis leaves and moreover, that the ABA-dependent transcription factor MYB96 is a key regulator of wax biosynthesis upon wounding. The data presented in this study led to the conclusion that wax is accumulating upon wounding to seal the wounding site, whereas TAG accumulation serves as storage for the acyl-moieties of the plastidial membrane lipids.

In addition it is shown, that MYB41, another ABA-dependent MYB transcription factor of the R2-R3 family-acts as a negative regulator of wax biosynthesis in Arabidopsis inflorescence stems. MYB41 suppresses the expression of the main fatty acid reductase in inflorescence stems, *CER4/FAR3*, and therefore reduces the amount of primary alcohols in the surface wax. A collection of *myb41* mutants helped to characterize regions in the MYB41 protein, which are important for its activity.

List of abbreviations

ABA	Abscisic acid
AAO	ABA oxidase
ABCG	Adenosine triphosphate binding cassette transporter G
ABI	Aba-insensitive
ACP	Acyl carrier protein
AOS	Allene oxide synthase
AP2	Apetala2
bp	Base pairs
Cas9	Clustered regularly interspaced short palindromic repeats associated protein 9
cDNA	Complementary DNA
CE	Collision energy
CER	*Eceriferum*
CLO3	Caleosin3
COI1	Coronatine insensitive 1
Col-0	Columbia 0
CRISPR	Clustered regularly interspaced short palindromic repeats
CYTB5	Cytochrome b5
DAG	Diacylglycerol
dde2-2	*Delayed dehiscence 2-2*
DEWAX	Decrease wax biosynthesis
DGAT	Acyl-coA:diacylglycerol acyltransferase
DGDG	Digalactosyldiacylglycerol
DP	Declustering potential
ECD	Enoyl-coA reductase
EP	Entrance potential
ER	Endoplasmic reticulum
EREBP	Ethylene responsive element binding protein
f. w.	Fresh weight
FA	Fatty acid
FAE	Fatty acid elongation
FAME	Fatty acid methyl ester
FAR	Fatty acyl-coA reductase
FAX1	Fatty acid export 1
FID	Flame ionization detector
GC	Gas chromatography
GPAT	Acyl-coA:glycerol-3-phosphate acyltransferase
gRNA	Guide RNA
HCD	Hydroxyacyl-coA dehydratase
hpw	Hours post wounding
JA	Jasmonic acid
JA-Ile	Jasmonoyl-isoleucine
JA-Leu	Jasmonoyl-leucine
JAR1	Jasmonic acid resistance 1
JAZ	Jasmonate inflorescence meristem domain protein
KCR	ß-keto acyl reductase
KCS	ß-keto acyl-coA synthase
LACS	Long-chain acyl-coA synthetases
LC-MS	Liquid chromatography coupled to mass spectrometry
LEC	Leafy-cotyledon
LOX	Lipoxygenase
LPAAT	Acyl-coA:lysophosphatidic acid acyltransferase
LTPG	Lipid transfer proteins G
MAH1	Midchain alkane hydroxylase
MeJA	Methyl jasmonate
MGDG	Monogalactosyldiacylglycerol
MPK6	Mitogen-activated protein kinase 6
MS	Mass spectometry
NADH/NAD+	Nicotinamide adenine dinucleotide
PA	Phosphatidic acid
PAH	Phosphatidic acid hydrolase
PAS2	Pasticcino2
PC	Phosphatidylcholine

PDAT	Phospholipid:diacylglycerol acyltransferase
PE	Phosphatidylethanolamine
PG	Phosphatidylglycerol
PP2C	2c-type protein phosphatase
PUFA	Polyunsaturated fatty acid
PYL	PYR1-like
PYR	Pyrabactin resistance1
qRT-PCR	Quantitative real-time polymerase chain reaction
RCAR	Regulatory component of ABA receptor1
ROP	Rho GTPases of plants
ROS	Reactive oxygen species
SA	Salicylic acid
SFR2	Sensitive to freezing
SnRK2	Snf1-related protein kinase 2
TAG	Triacylglycerol
TLC	Thin-layer chromatography
UPLC	Ultra performance liquid chromatography
v/v	Volume per volume
VLCFA	Very long chain fatty acids
WIN1/SHN1	Waxinducer1/shine1
WRI	Wrinkeled1
WSD	Bifunctional wax ester synthase/diacylglycerol acyltransferase
WT	Wild type

Chapter 1: Introduction

1.1 Plant Lipids

Lipids are essential components of a plant cell. They serve as energy storage, signaling molecules, structural components of membranes as well as hydrophobic barriers. Lipids can be divided into polar and neutral lipids based on their hydrophobicity. Polar lipids harbor non-polar fatty acid moieties as well as at least one polar head group e.g. a galactose or a phosphate group. They make up almost all lipids present in green parts of plants. To polar lipids belong: phospholipids and glycolipids. The second group of lipids are neutral lipids, which by definition lack the polar head group. They can serve as storage compounds, like triacylglycerol (TAG), membrane components (sterol esters) and hydrophobic coatings for plant tissues as cutin, suberin and wax.

1.1.1 Fatty acid biosynthesis in plants

Most lipids (except sterols) harbor fatty acids (FA). They consist of one carboxyl group at the end of a linear hydrocarbon chain of at least four carbons. The most abundant FA in plants contain 16 or 18 carbons in their chains, however very long chain fatty acids (VLCFA) consisting of 20 or more carbons are found in sphingolipids or build up hydrophobic barriers such as the cuticle or the suberin layer, which cover the above and below ground parts of a plant. FA biosynthesis starts in plastids. The first step of this process is the ATP-dependent carboxylation of acetyl-CoA to form malonyl-CoA. Afterwards, in a cyclical reaction malonyl-CoA is first added to acetyl-CoA and later to the growing chain. In each cycle one molecule of CO_2 is lost leading to the addition of two-carbon units during each round. This process stops at a chain length of C16 or C18. Modification of FA by desaturation, further elongation or usage by acyltransferases may take place in the envelope of plastids or mitochondria as well as in the membrane of the endoplasmic reticulum (ER). FA synthesized in plastids are attached to the acyl carrier protein (ACP). Their export requires the release from ACP by an acyl-ACP thioesterase and activation to CoA-thioesters by long-chain acyl-CoA synthetases (LACS) (Li-Beisson et al., 2010). Acyl-CoAs with a chain length of C16-C18 are

exported from plastids and can be portioned between further modifications (e.g. elongation in chapter 1.4.1.1) or directed into the lipid biosynthesis.

1.2 Polar Lipids

Polar lipids may be based on a glycerol backbone, polar head group and non-polar fatty acid moieties. There can be two types of polar group attached to glycerol backbone: phosphate or sugar harboring groups. The first group defines phospholipids, which may consist only of a phosphate group (phosphatidic acid, PA) or a phosphate ester, such as choline (phosphatidylcholine, PC), ethanolamine (phosphatidylethanolamine, PE), serine (phosphatidylserine, PS) or glycerol (phosphatidylglycerol, PG). Phospholipids are present in almost all membranes, where they can form a lipid bilayer. However, PG is normally only found in plastidial and mitochondrial membranes. The second group of polar lipids consist of galactolipids. They contain either a galactose, a glucoronate or a sulfoquinovate group attached to the glycerol backbone. They may contain one galactose (monogalactosyldiacylglycerol, MGDG) or two (digalactosyldiacylglycerol, DGDG). Like PG, MGDG and DGDG are very abundant in plastidial membranes and are integral elements of photosynthetic complexes (Li-Beisson et al., 2010; Hölzl and Dörmann, 2019).

1.3 Triacylglycerols as storage lipids

TAG is composed of three FA linked via an ester bond to the glycerol backbone. They are mostly present in seeds, serving as a storage form of carbon to support germination and seedling growth. In adult plants TAG are essential for proper development and growth. They rarely occur in vegetative plant organs under normal conditions however they play a key role in lipid homeostasis (more in chapter 1.5.3).

1.3.1 TAG biosynthesis

The TAG biosynthesis pathway, a side-branch of the Kennedy pathway, named after one of its discoverers (Weiss et al., 1960), starts with the esterification of acyl-CoA and glycerol-3-phosphate by acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) in the *sn*-1 position (Figure 1), where lysophosphatidic acid (LPA) is formed. In the Arabidopsis genome nine genes are coding

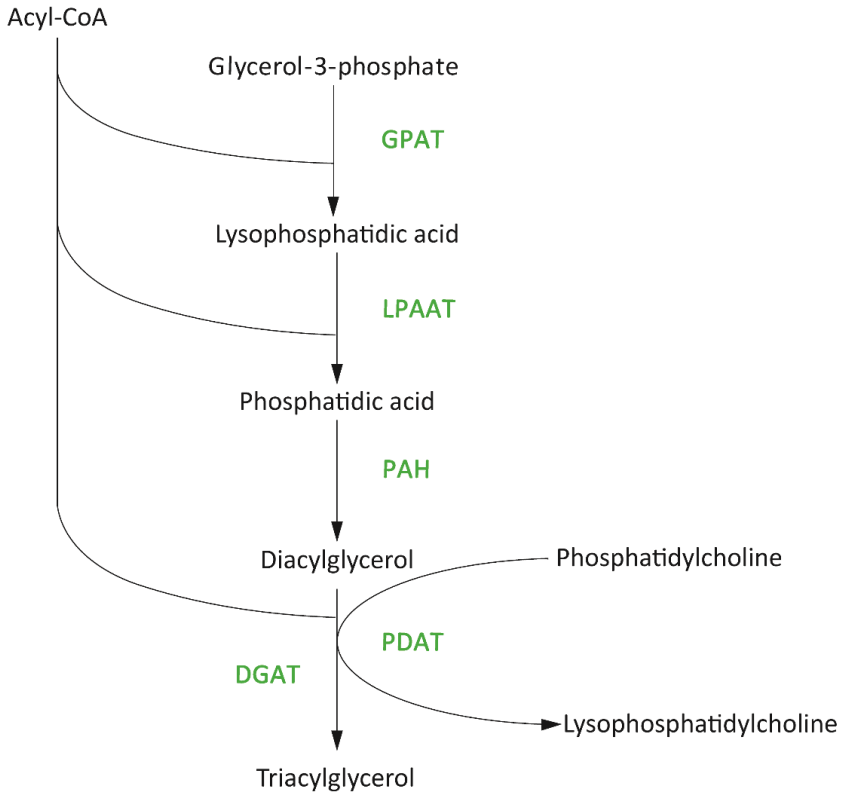

Figure 1. The triacylglycerol biosynthesis pathway.
Glycerol-3-phosphate is acylated by the acyl-CoA:glycerol-3-phosphate acyltransferase (GPAT) to obtain lysophosphatidic acid (LPA). Afterwards, phosphatidic acid (PA) is formed by the acylation of LPA with another acyl-CoA with acyl-CoA:lysophosphatidic acid acyltransferase (LPAAT). PA hydrolase (PAH) removes a phosphate group of PA and this leads to formation of diacylglycerol (DAG). DAG can be again acylated either by acyl-CoA:diacylglycerol acyltransferase (DGAT) or by phospholipid:diacylglycerol acyltransferase (PDAT) which is transferring an acyl-moiety from phosphatidylcholine onto DAG.

for GPAT enzymes, however only one of them, *GPAT9,* is involved in TAG biosynthesis (Shockey et al., 2015). To the *sn*-2 position of the LPA, another acyl-CoA is attached by acyl-CoA:lysophosphatidic acid acyltransferase (LPAAT), which results in formation of PA. In

Arabidopsis two LPAAT enzymes (LPAAT2 and LPAAT3) are confirmed to play a role in TAG formation (Kim and Huang, 2004; Kim et al., 2005). The phosphate group of PA may be removed by phospatidate phosphatases, in Arabidopsis phosphatidic acid hydrolase (PAH), PAH1 and PAH2. This leads to the formation of diacylglycerol (DAG) (Eastmond et al., 2010). The last acylation in the sn-3 position of DAG is catalyzed by acyl-CoA:diacylglycerol acyltransferase (DGAT). There are three DGAT enzymes known in Arabidopsis. However, only one of them, DGAT1, plays an essential role in TAG biosynthesis (Katavic et al., 1995; Jako et al., 2001). Two other enzymes: DGAT2 and DGAT3 can produce TAG, but their activity is much lower (Aymé et al., 2014; Aymé et al., 2018). The acylation of DAG can be also catalyzed by phospholipid:diacylglycerol acyltransferase (PDAT; in Arabidopsis PDAT1). Here, the acyl moiety is directly transferred from a phospholipid to a DAG molecule (Dahlqvist et al., 2000). Newly synthesized TAG are stored in lipid droplets, lipid rich organelles surrounded by a phospholipid monolayer. Extensive TAG biosynthesis occurs throughout seed development. Besides that, this process also takes place during cell death or in response to stress in Arabidopsis leaves (for TAG biosynthesis upon stress see chapter 1.5.3).

1.3.2 Regulation of TAG biosynthesis

Since extensive TAG biosynthesis occurs mostly in developing seeds, research on the regulation of this process is mostly focused in this organ. TAG formation can be induced by several factors like nitrogen deprivation, sucrose or salt treatment (Martin et al., 2002; Gaude et al., 2007; Yang et al., 2011). In addition, many phytohormones are involved in the regulation of the TAG biosynthesis: cytokinins, gibberellic acid, auxins, ethylene or abscisic acid (ABA) and only slightly salicylic acid (SA) and jasmonic acid (JA) (Yang et al., 2011; Kong et al., 2013). The first discovered transcription factor that positively regulates TAG biosynthesis is WRINKELED1 (WRI1) (Cernac and Benning, 2004; To et al., 2012). WRI1 belongs to the APETALA2 (AP2)/ethylene responsive element binding protein (EREBP) family of transcription factors. It does not directly regulate enzymes from the Kennedy pathway, but those from FA biosynthesis (Ruuska et al., 2002). Additionally, transcription factors that increase TAG biosynthesis are LEAFY-COTYLEDON1 and 2 (LEC1 and LEC2). They belong to the B3 transcription factor family. The B3 DNA binding domain is

formed by seven β-strands and two α-helices that can interact with the DNA (Swaminathan et al., 2008). Overexpression of LEC1 or LEC2 results in TAG accumulation in leaves and higher expression of genes involved in FA biosynthesis (Santos Mendoza et al., 2005; Mu et al., 2008). Moreover, it was shown that LEC2 can positively regulate expression of *WRI1* (Baud et al., 2007). A transcription factor that directly regulates one of the enzymes from TAG biosynthesis is ABA-Insensitive 4 (ABI4). It also belongs to the B3 transcription factor family and it binds directly to the promotor of *DGAT1* (Yang et al., 2011). Moreover, it was proposed that ABI4 and another ABI transcription factor, ABI5, are synergistically regulating TAG biosynthesis in Arabidopsis seedlings. Both ABI4 and ABI5 play important roles in ABA-dependent seed germination (Finkelstein et al., 1998; Finkelstein and Lynch, 2000). It was recently found that one of the MYB transcription factors, MYB96, is regulating TAG biosynthesis (more about this transcription factor in chapter 1.4.3). MYB96 binds directly to the promotor of *PDAT1* and indirectly regulates the expression of *DGAT1* (Lee et al., 2018). Mutant plants lacking MYB96 have a reduced amount of TAG in their seeds. Interestingly, MYB96 is directly controlling the expression of *ABI4*. It was suggested that combined MYB96 and ABI4 signaling is important for regulating seed germination (Figure 2) (Lee et al., 2015).

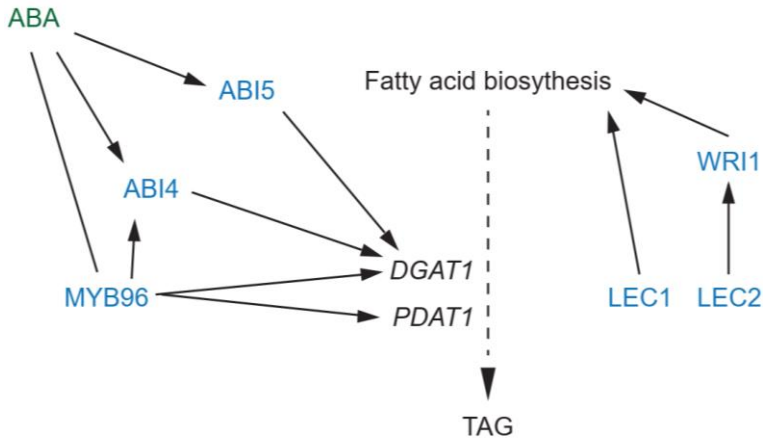

Figure 2. The TAG biosynthesis is regulated by different sets of transcription factors.
The transcription factors LEC1 and LEC2 regulate FA biosynthesis via WRI1 and in consequence TAG biosynthesis. The ABA-dependent transcription factors ABI4, ABI5 and MYB96 can directly regulate the expression of *DGAT1* or/and *PDAT1*, genes coding enzymes from the last part of TAG biosynthesis. Moreover, MYB96 binds directly to the promotor of *ABI4.*

1.4 Cuticular wax as a lipid barrier

The adaptation to a non-aqueous environment by the first land plants required the construction of a hydrophobic barrier protecting themselves from desiccation, UV radiation and organ fusion. One such a barrier is the cuticle which is already present in the first land plants, the bryophytes (Cook and Graham, 1998). The cuticle is covering all aerial plant organs, except of the stems of woody plants, and it is composed of cutin and wax. Cutin is a FA and glycerol based polyester forming the scaffold of the cuticle (Fich et al., 2016). Whereas wax is a combination of aliphatic compounds surrounding and covering cutin and therefore the plant surface. Moreover, wax can also be present as epicuticular wax in form of crystal-like structures (Figure 3) (Samuels et al., 2008).

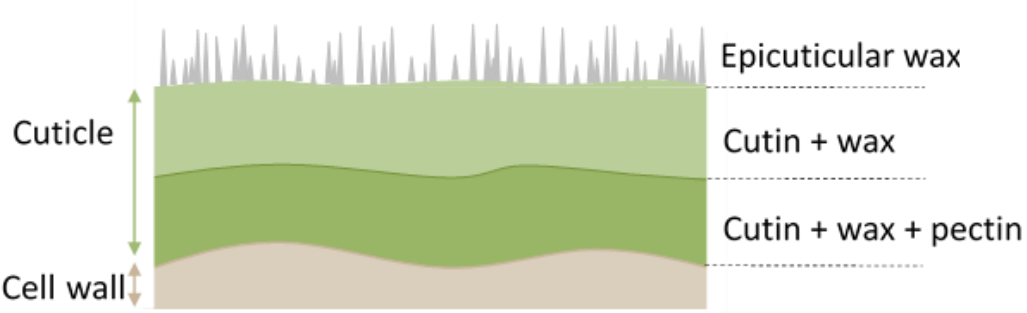

Figure 3. The cuticle is composed of cutin and wax.
Together with pectin, they form a layer covering the cell wall. On top of this layer, cutin and wax are sealing the plants surface. The outermost structure consists of epicuticular wax, which forms crystal-like structures.

Together with cutin, they serve as first barrier protecting plants from environmental threats. Developing of such a defense layer was an important part of land colonization by plants. The cuticle provides not only a first layer of resistance towards pathogens and insects, but serves also as an excellent barrier to prevent loss of water. Moreover, the waxy surface reflects the light and phenolic compounds in the cutin structure are absorbing radiation (Koch et al., 2009). Understanding cuticle properties, its biosynthesis as well as the regulation of the biosynthesis, can help to improve its function and therefore increase the plant resistance to desiccation or pathogens.

1.4.1 Wax biosynthesis

The discovery of the wax biosynthesis pathway in *A. thaliana* started in the 1970s by a reverse genetic approach resulting in a mutant collection called *eceriferum* (Latin for wax-less) (Dellaert et al., 1979). This screen helped to identify 21 *ECERIFERUM* (*CER*) genes, in which mutations caused glossy stem phenotypes suggesting lower wax content on the stem surface (Koornneef et al., 1989). More genes of the wax pathway were identified by co-expression and co-regulation data that make *A. thaliana* a well suited model organism also for wax biosynthesis. In Arabidopsis leaves, wax consists of alkanes (c. 55 %), primary alcohols (c. 20 %), aldehydes (c. 15 %), VLCFA (c. 10 %) and wax esters (< 1 %). In inflorescence stems and siliques however, the major components are ketones and secondary alcohols (Lewandowska, unpublished). Moreover, inflorescence stems of Arabidopsis are covered with c. 30 times more wax than leaves and 20 times more than siliques.

1.4.1.1 Fatty acid elongation

Wax biosynthesis takes place in epidermal cells. It starts with the elongation of saturated FA of 16 and 18 carbons coupled to CoA. The saturated FA are derived from the *de novo* FA synthesis in the plastids. The fatty acid elongation (FAE) is carried out by a multienzyme complex located in the ER membrane. VLCFA are formed by four enzymatic reactions resulting in a two-carbon extension of the acyl chain in every cycle. The multienzyme complex performing this reaction consists of the following enzymes: condensing enzyme (ß-keto acyl-CoA synthase, KCS), reducing enzyme (ß-keto acyl reductase, KCR), dehydratase (hydroxyacyl-CoA dehydratase, HCD) and enoyl-CoA reductase (ECR) (Figure 4)(Samuels et al., 2008). The KCS enzymes are determining the specificity of each elongation step. In the Arabidopsis genome, 21 genes coding for KCS enzymes are present. However, only 7 of them are wax specific: KCS1 (Todd et al., 1999), KCS2 and KCS20 (Lee et al., 2009), KCS9 (Kim et al., 2013), KCS10 (Lolle et al., 1997; Pruitt et al., 2000), KCS16 (Hegebarth et al., 2017) and KCS6/CER6 (Millar et al., 1999; Fiebig et al., 2000). Furthermore, KCS6/CER6 seems to be a key enzyme for the wax biosynthesis in Arabidopsis, since a mutant of the gene coding for this enzyme exhibited a strong reduction in wax content on leaves and inflorescence stems surface (Fiebig et al., 2000; Hooker et al., 2002).

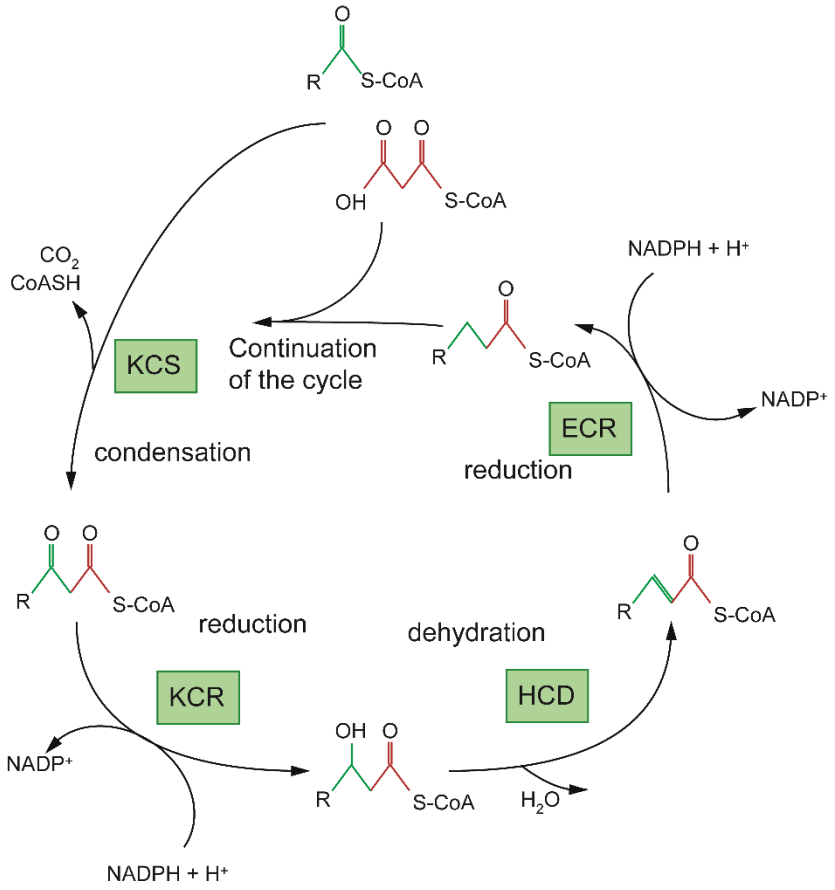

Figure 4. The fatty acid elongation complex in the endoplasmic reticulum generates very long chain fatty acids.
To acyl-CoA of 16 or 18 carbons (green) a malonyl-CoA is condensed (red) by the condensing enzyme (ß-keto acyl-CoA synthase, KCS) and Coenzyme A (CoA) and CO_2 are released. Next, the reduction reaction takes place, where the 3-ketone group is reduced to a 3-hydroxyl group by the reducing enzyme (ß-keto acyl reductase, KCR) and a reduction of NADPH to $NADP^+$. Afterwards, the dehydration takes place by a dehydratase (hydroxyacyl-CoA dehydratase, HCD). The last reaction is a reduction of a double bond and the formation of an acyl-CoA with two additional carbons. This step is catalyzed by the enoyl-CoA reductase (ECR). Acyl-CoA elongated by two carbons can enter the cycle again for further elongation. FA are synthesized via the same mechanism, however the substrates are attached to ACP – acyl carrier protein – instead of acyl-CoA and this synthesis take place in plastids.

Previous research on FAE in yeast helped to identify KCR enzymes in Arabidopsis. KCR1 and KCR2 in Arabidopsis were found based on sequence similarity to the beforehand identified yeast KCR enzyme (Beaudoin et al., 2002). However, only KCR1 is required for FAE in Arabidopsis, since a mutant of KCR2 exhibits no wax reduced phenotype (Beaudoin et al., 2009). Another example of transferring knowledge from yeast research is the discovery of a HCD enzyme named

PASTICCINO2 (PAS2) in Arabidopsis. PAS2 was known before as a regulator of cell proliferation and differentiation (Faure et al., 1998; Bellec et al., 2002; Harrar et al., 2003). Its role in wax biosynthesis was not known until the discovery of the HCD enzyme in yeast. That led to the identification of PAS2 as a HCD enzyme of the FAE complex in Arabidopsis (Bach et al., 2008). Moreover, PAS2 physically interacts with CER10, an ECR enzyme and the last enzyme of the FAE pathway (Zheng et al., 2005; Bach et al., 2008). CER10 is the only characterized ECR enzyme so far. However, a mutant of CER10 shows only a reduced wax amount of 60 %., suggesting that there must be another ECR enzyme present in Arabidopsis. Another enzyme taking part in FAE is CER2, though it does not belong to any of the FAE complex enzyme families. CER2 is a BAHD acyltransferase and the mutant lacking this enzyme does not produce VLCFA longer than 28 carbons. It suggests that CER2 is another important enzyme taking part in FAE (Haslam et al., 2012).

1.4.1.2 Alcohol- and alkane-forming pathways

VLCFA of 26-36 carbons can be either directly exported outside to the plant surface or channeled within the cell into two pathways, the alkane and/or alcohol-forming pathway (Figure 5). In the latter pathway, VLCFA can be reduced to primary alcohols by fatty acyl-CoA reductase (FAR). In Arabidopsis the main wax-specific reductase in inflorescence stems is CER4/FAR3, however the mutant of this protein still produces alcohols of 30 carbons, suggesting that there might be another FAR specific for the production of fatty alcohols longer then C28 (Rowland et al., 2006). One of the candidates could be *FAR6* (At3g56700), which is highly expressed in epidermal cells of inflorescence stems (Suh et al., 2005). Primary alcohols produced in the alcohol-forming pathway are even-numbered from 22 up to 34 carbons. They can be right away transported to the cell surface or can be esterified to acyl-CoA by a bifunctional wax ester synthase/diacylglycerol acyltransferase (WSD) to form wax esters (Li et al., 2008). There are 11 genes coding for WSD enzyme in Arabidopsis, however only *WSD1* shows a main impact on wax ester biosynthesis (Li et al., 2008; Patwari et al., 2019).

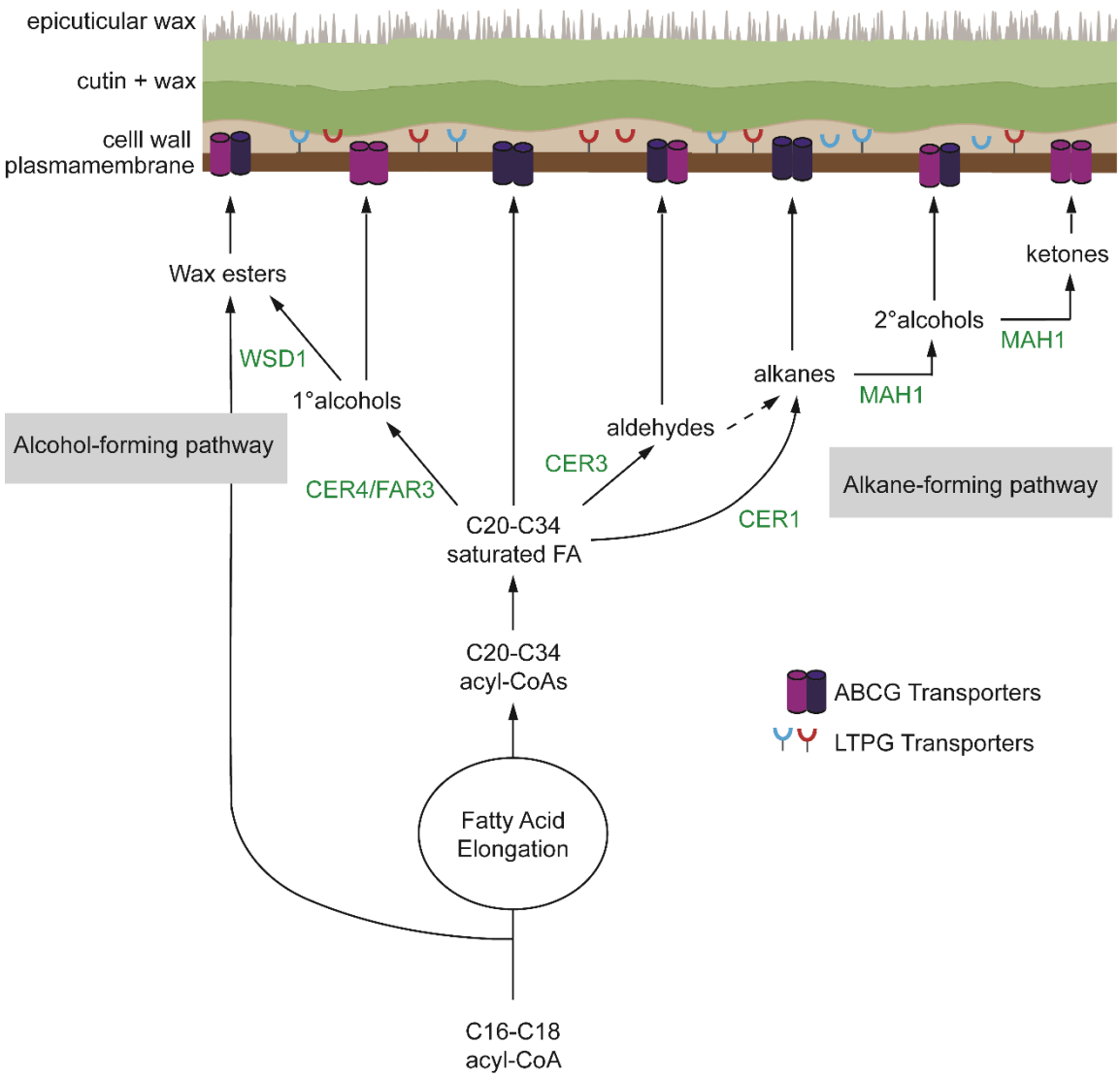

Figure 5. Simplified scheme of wax biosynthesis.

The pathway starts with the elongation of C16 – C18 acyl-CoAs (see figure 4). Afterwards elongated fatty acids (FA) are either directly transported through the plasma membrane and cell wall or enter the alcohol-forming pathway or alkane-forming pathway. In the alcohol-forming pathway, FA are reduced to primary alcohols by fatty acyl-CoA reductase (FAR). The main FAR in Arabidopsis is CER4/FAR3. Primary alcohols can be either exported outside of the cell or esterified with C16-C18 acyl-CoAs to form wax esters. This esterification is catalyzed by a bifunctional wax ester synthase/diacylglycerol acyltransferase (WSD). The main one in Arabidopsis is WSD1. In the alkane-forming pathway, aldehydes and alkanes from VLCFA are synthesized by the enzymes CER3 and CER1. In inflorescence stems and siliques, alkanes can be further modified to obtain secondary alcohols and ketones by the enzyme midchain alkane hydroxylase (MAH1). VLCFA, primary alcohols, wax esters, alkanes, aldehydes, secondary alcohols and ketones are transported through the plasma membrane by adenosine triphosphate binding cassette transporters family (ABCG), which can form homo- and heterodimers, or by lipid transfer proteins (LTPG).

In the alkane-forming pathway alkanes, aldehydes, ketones and secondary alcohols are produced from VLCFA. There is no clear mechanism known how alkanes are synthesized. It was proposed that alkanes are produced via the formation of aldehydes as intermediates, however this hypothesis was never proven in *planta* (Dennis and Kolattukudy, 1991; Schneider-Belhaddad and Kolattukudy, 2000). The hypothesis is based on the decarbonylation mechanism that releases a CO_2 during the conversion of aldehydes to alkanes. This idea is further supported by the fact, that aldehydes present in wax are even-numbered (from 26 to 34 carbons) and alkanes are odd-numbered (from 25 to 35 carbons). More experimental data is needed to fully understand this part of the pathway. However, enzymes having a main impact on alkane biosynthesis are known. A mutant of *CER1* exhibits a dramatic decrease in alkanes (Aarts et al., 1995), while a mutant of *CER3* shows a dramatic decrease of alkanes and aldehydes (Chen et al., 2003). It was proposed that CER3 may produce aldehydes by the reduction of VLCFA, while CER1 is producing alkanes from aldehydes. Moreover, both of these proteins interact with each other and with an ER-localized cytochrome b5 (CYTB5) which can serve as a redox cofactor (Bernard et al., 2012). In inflorescence stems and siliques, the most abundant alkane (with 29 carbons) is oxidized to obtain secondary alcohols and further oxidation leads to the formation of ketones. Both of those reactions are catalyzed by midchain alkane hydroxylase 1 (MAH1). The lack of this enzyme in Arabidopsis leads to a deficiency in ketones and secondary alcohols and an increase in the amount of alkanes (Greer et al., 2007).

1.4.1 Transport of wax out of the cell

The wax component from both, the alkane- and alcohol-forming pathways as well as VLCFA are exported to the plant surface. Firstly, wax components have to be transferred from the ER to the plasma membrane across the hydrophilic environment of the cytoplasm. This part of wax assembly and transport is the least known. Up to date, there were many hypotheses proposed, however none of them were proven experimentally. It is suggested that wax components can be transported to the plasma membrane by soluble acyl carrier proteins, accumulating in lipid droplet-like vehicles, via the trans-Golgi network or by using the contact sites between the ER and

plasma membrane. When wax components reach the plasma membrane, they have to be secreted into the extracellular matrix (Bernard and Joubès, 2013; McFarlane et al., 2017). The first discovered wax transporter was CER5/ABCG12 (Pighin et al., 2004). It belongs to the peroxisomal adenosine triphosphate binding cassette transporter (ABC) family and to the half transporter subfamily ABCG, which have to oligomerize to form a functional transporter. A mutant of CER5/ABCG12 contains a total wax amount equal to wild type (WT) plants, but the epidermal peel analysis revealed that the mutant has a significantly lower wax amount on the surface. Moreover, this mutant contained lipidic inclusions in the cell (Pighin et al., 2004). After the discovery of the first transporter, many others were identified based on the similarity to ABCG12 and on expression studies. Another ABC transporter, ABCG11, was found to be involved in wax, cutin and suberin transport (Bird et al., 2007; Panikashvili et al., 2007). Furthermore, ABCG11 physically interacts with itself and with ABCG12 and since they are both half-transporters, they may form homo- or heterodimers (McFarlane et al., 2010). Another family of proteins taking part in wax export are lipid transfer proteins (LTP). Their expression is highly enriched in the epidermal peel of inflorescence stems. Mutants of *LTPG1* and *LTPG2* have a reduced wax amount on their inflorescence stems surface (Kim et al., 2012). All described wax transporters were identified in inflorescence stems of Arabidopsis. The transporter mutants do not show strong alterations in their wax amount on leaves. Due to the lack of an experimental approach to analyze wax transporters in Arabidopsis leaves, which cannot be peeled due to their thickness, transporters specific for leaves remain unknown.

1.4.2 Regulation of wax biosynthesis

Wax biosynthesis is tightly regulated during the plant development and in response to changes in the environment. The majority of the genes coding for enzymes of the wax pathway show higher expression in young developing organs. Their expression is enriched in the top of young inflorescence stems, which is the main wax-synthesizing organ (Suh et al., 2005). The wax biosynthesis can be controlled at transcriptional, post-transcriptional or post-translational level (Figure 6). There are many transcription factors known, which regulate the expression of genes involved in wax biosynthesis directly or indirectly. The first one discovered is

WAXINDUCER1/SHINE1 (WIN1/SHN1). It was named due to the phenotype of its overexpression line - the leaves had a shiny appearance - which was a result of an enhanced wax accumulation. WIN1/SHN1 belongs to the AP2/EREBP family of transcription factors. WIN1/SHN1 is regulating genes from the alkane-forming pathway (*CER1*) but also from FAE (*CER2*). Moreover, the overexpression of other genes belonging to the same clade, *SHINE2* and *SHINE3,* leads to the same phenotype as the overexpression of *WIN1/SHN1*, meaning that probably the whole clade is involved in regulating wax biosynthesis (Aharoni et al., 2004; Broun et al., 2004). However, further research revealed that WIN1/SHN1 is also involved in the regulation of cutin biosynthesis. Overexpression of *WIN1/SHN1* leads to higher expression of genes involved in cutin biosynthesis and increased amount of cutin monomers (Kannangara et al., 2007). A second family of transcription factors that regulate wax biosynthesis are MYB transcription factors. They are characterized by a highly conserved MYB-binding domain, which consists of three α-helices. In Arabidopsis almost 200 MYB transcription factors can be found. MYB transcription factors can be divided into 4 classes. The MYB transcription factors regulating wax biosynthesis belong to the first class of MYB transcription factors characterized by two DNA binding domains (R2 and R3) located close to the N-terminus of the protein. This R2R3 class is the most abundant class of MYB transcription factors (Dubos et al., 2010). The first, and most characterized, transcription factor from this family, regulating wax biosynthesis is MYB96. This transcription factor, while overexpressed, is enhancing plants resistance to drought stress, whereas plants lacking MYB96 are more susceptible to desiccation (Seo et al., 2009). MYB96 plays a similar role in *Camelina sativa*, since its overexpression confers drought resistance (Lee et al., 2014). MYB96 is ABA dependent, since external application of ABA induced its expression, but it is acting independently from JA (more about roles of those two phytohormones in chapters 1.5.1.2 and 1.5.2). The drought tolerance in the overexpression lines of *MYB96* was connected to the very high amount of surface wax on leaves and inflorescence stems. Consequently, high susceptibility to desiccation of *myb96* mutant was a result of a 25 % reduction of the wax content on the surface of leaves and inflorescence stems, especially in the products of the alkane-forming pathway and in the amount of VLCFA (Seo et al., 2011). Moreover, this reduction of wax on the leaf surface might lead to higher susceptibility to pathogens as has been shown for *Pseudomonas syringae* (Seo and Park, 2010). MYB96 is also partially responsible for inducing wax biosynthesis upon drought, since

mutant of *MYB96* show a reduced wax accumulation in this condition (Seo et al., 2011). MYB96 is not only a part of the regulation of wax biosynthesis but it is also involved in the regulation of the circadian clock (Lee et al., 2016), stimulating FAE in seeds (Lee et al., 2015), regulating seed dormancy and seed germination (Lee et al., 2015; Lee and Seo, 2015) as well as TAG biosynthesis (Lee et al., 2018). It was experimentally shown that MYB96 might bind directly to the promotors of the following genes from the wax pathway: *CER6/KCS6, KCR1, CER2, CER1, CER3* and *WSD1* (Seo et al., 2011). Another transcription factor sharing 70 % similarity in its amino acid sequence with MYB96 is MYB94. While MYB96 is mostly controlling the alkane-forming pathway, MYB94 is, additionally regulating the alcohol-forming pathway. It binds to the promotors of the same genes as MYB96, but also to the promotor of the gene coding for the major FAR, *CER4/FAR3* (Lee and Suh, 2014). Moreover, MYB94 can partially complement a lack of MYB96 and both of those transcription factors are additively regulating wax biosynthesis during drought. Together, they regulate approximately 40 % of the wax biosynthesis (Lee et al., 2016). Research on MYB94 led to the identification of a putative activation domain in MYB transcription factors, which is located at its C terminus (Lee and Suh, 2014). It was also found that MYB30 is involved in wax biosynthesis in Arabidopsis. MYB30 is well-known in the plant immunity field and it is a positive regulator of programmed cell death (Vailleau et al., 2002). Overexpression of MYB30 leads to a high expression of genes related to VLCFA biosynthesis and to an elevated wax amount. Some of the genes, like *CER10, CER2, PAS2, CER3* were up-regulated in WT during pathogen infection (*Xanthomonas campestris*) and this effect was enhanced in lines overexpressing MYB30 (Raffaele et al., 2008). Interestingly, both MYB30 and MYB96 are ubiquitinated by the same RING-type E3 ligase, MIEL1, which is negatively regulated by ABA (Lee and Seo, 2016). The last described MYB transcription factor involved in wax biosynthesis so far is MYB41. It was shown, that MYB41 is responsive to ABA, desiccation and salt stress (Cominelli et al., 2008). Its overexpression leads to the formation of suberin like structures on the leaf surface and a higher permeability of the cuticle (Lippold et al., 2009; Kosma et al., 2014). Moreover, Arabidopsis plants overexpressing MYB41 are producing more wax on their leaf surface, however that was only proven in one overexpression line (Kosma et al., 2014). Another study revealed that MYB41 needs to be phosphorylated by mitogen-activated protein kinase 6 (MPK6) to be active and that this reaction takes place on a serine (Ser^{251}) located at the C-terminus of the protein (Hoang et al., 2012). The

full function of this transcription factor remained unknown, also due to the fact, that a loss-of-function mutant of MYB41 was so far not available. Up to date, there are five positive regulators of wax biosynthesis known (WIN1/SHN1, MYB96, MYB94, MYB30 and MYB41). However, most likely there are more of them present in Arabidopsis. Interestingly, overexpression of any of these positively regulating transcription factors results in a dwarf phenotype in Arabidopsis. This shows how important a tight regulation of the wax biosynthesis is, where even a small increase in its amount leads to serious morphological defects. Therefore, in addition to the positive regulators, there are also two negative regulators of the wax biosynthesis known in Arabidopsis - DECREASE WAX BIOSYNTHESIS 1 and 2 (DEWAX1 and DEWAX2) (Go et al., 2014; Kim et al., 2018). Both belong to the same family as WIN1/SHN1 – AP2/EREBP. *DEWAX1* is highly expressed during an extended dark period, which correlates with reduced wax amount in plant exposed to 5 days with no light. A mutant of *DEWAX1* has more wax on the surface of leaves and inflorescence stems, while the opposite effect was shown for plant overexpressing this protein. DEWAX1 binds to the promotors of *CER1, FAR6* and *CER10* (Go et al., 2014). However, no dark-stress experiment was performed with DEWAX1 loss- or gain-of-function lines, so its direct function in negatively regulating wax biosynthesis during dark is not fully proven. Nevertheless, it was shown that lines overexpressing DEWAX1 in Arabidopsis and Camelina are more resistant to a necrotrophic fungus (*Botrytis cinerea)* (Ju et al., 2017). A screen for genes being enriched in the epidermis of inflorescence stems led to the identification of DEWAX2. Its amino acid sequence is 70 % identical with DEWAX1. DEWAX2 loss- or gain-of-function lines are showing a similar phenotype as the one of DEWAX1. Moreover, DEWAX2 also binds to promotor of *CER1* (Kim et al., 2018).

The only protein involved in controlling wax biosynthesis on a post-transcriptional level is CER7. It acts as a 3'-5' exoribonuclease and is part of the RNA-processing/degrading exosome complex. CER7 is involved in the regulation of *CER3* expression by controlling post-transcriptional silencing driven by small interfering RNAs (Hooker et al., 2007; Lam et al., 2012). Up to date, only one factor is known which regulates wax biosynthesis post-translationally - CER9. CER9 is predicted to be a RING-type E3 ligase. A mutant of *CER9* accumulated 13 times more VLCFA on the leaf surface compared to control plants, while other wax components were strongly reduced (Lü et al., 2012). However, in inflorescence stems the mutant had much more alcohols and FA with a reduction of the products of the alkane-forming pathway. This result shows that wax biosynthesis is

differentially regulated in the particular organs and at many steps of their biosynthesis. Moreover, as expected, mutants of CER9 were more resistant to desiccation, since their total wax and cutin amount was higher (Lü et al., 2012). Interestingly, it was later shown that CER9 is also involved in ABA signaling in seeds and seedlings (Zhao et al., 2014). Wax formation can be regulated at each step of its biosynthesis, however almost all regulators mentioned above are dependent on ABA, which is a key regulator of wax biosynthesis (more about role of ABA in wax biosynthesis in chapter 1.5.2).

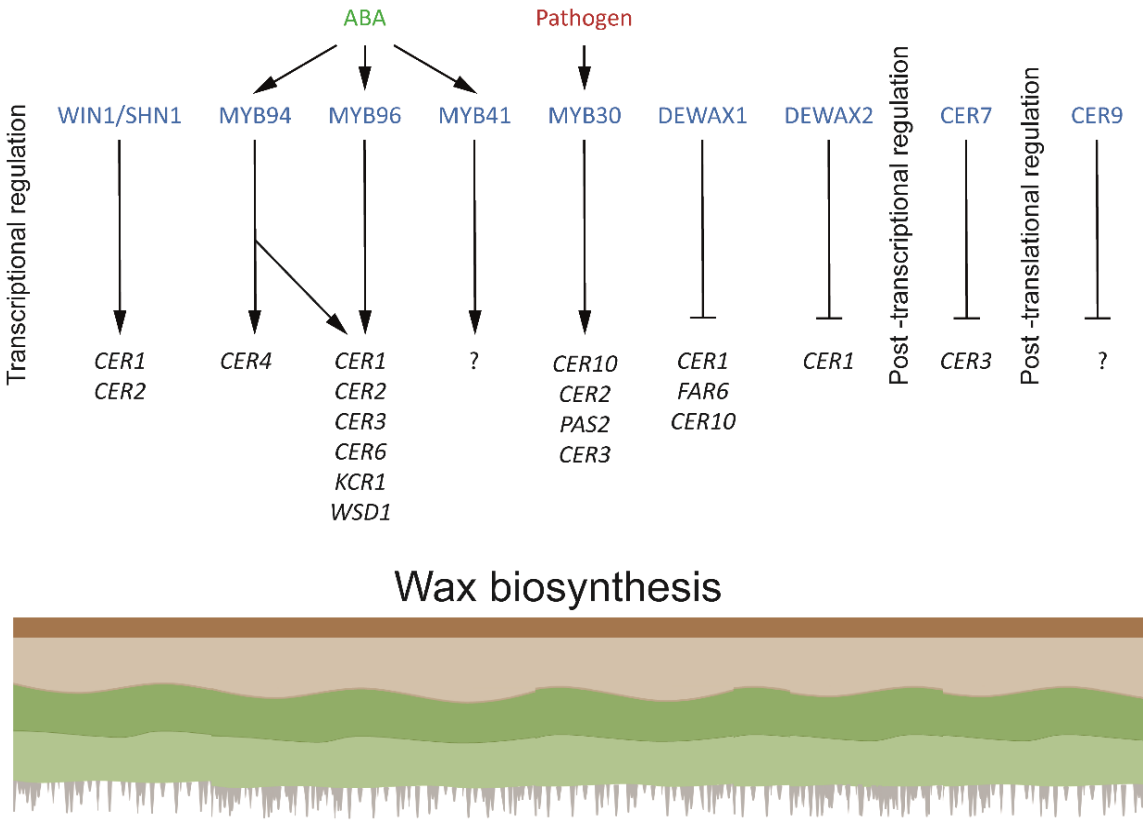

Figure 6. Overview of the transcription factors being involved in the regulation of wax biosynthesis. Transcription factors, which act as positive regulators of the wax pathway are WIN/SHN1, MYB94, MYB96, MYB41, MYB30, while DEWAX1 and DEWAX2 are negative regulators. MYB94, MYB96 and MYB41 are controlled by ABA whereas MYB30 is induced upon pathogen attack. CER7 is regulating the expression of *CER3* post-transcriptionally and CER9 is regulating wax biosynthesis post-translationally.

1.5 Plant stress response and lipid metabolism

Plants face threatening factors during their lifetime. Those factors can be divided into biotic stresses caused by living organisms, and abiotic stresses like drought, heat, cold, lack of nutrients etc. After plants colonized land, they had to adapt to the changed living conditions and to develop mechanisms to cope with stress factors. Many researchers focus nowadays on the plants stress response, to better understand those mechanisms, and in the consequence to improve the adaptation of plants to stress conditions, especially in times of climate change.

1.5.1 Plant response to wounding and feeding insects

Wounding is one of the most common plant threats. It can be caused by wind, strong rain, feeding insects, grazing and trampling animals or by mechanical damage. Wounded tissues undergo many metabolic changes to defend themselves from damages and to start regeneration processes. One of the earliest responses of a plant cell to wounding is the accumulation of Ca^{2+} in the cytosol and the production of reactive oxygen species (ROS) (Orozco-Cardenas and Ryan, 1999). Both act as signaling molecules in the defense response (Orozco-Cardenas et al., 2001; L'Haridon et al., 2011; Baxter et al., 2013; Beneloujaephajri et al., 2013). Moreover, upon wounding, many alterations are observed in the plants transcriptome, which is adapting to the stress conditions via a precisely working signaling network. Plant phytohormones are produced as wound signals and the formation of secondary metabolites as well as the remodeling of lipids are part of the defense program and the regeneration machinery (Vu et al., 2014; Vu et al., 2015). Under laboratory conditions, mechanical wounding is a model for mimicking insect attack, nevertheless it mimics only the physical plant-insect interaction, but not the response to oral secretions by insects.

1.5.1.1 Wax is the first layer of defense

The role of wax as a barrier for insect attack was studied extensively for the last 100 years. Wax is involved in many aspects of the plant-insect interaction. They help to trap insects in carnivorous

plants or prevent ants from removing pollen by forming a slippery surface on inflorescence stems. However, most studied is the mechanism, that surface wax is preventing adhesion of insects (Federle and Endlein, 2004; Gaume et al., 2004). Several studies have shown that less insects are feeding on glacious leaves, which have a higher surface wax load, than on glossy ones with less wax of different cultivars of *Eucalyptus* and *Brassica oleracea* (Edwards, 1982; Stoner, 1990). Scanning electron microscopy revealed that on wax-crystal rich structures, insects cannot attach so well (Stork, 1980). Moreover, a higher wax amount increases the anti-adhesive properties, which reduces the attachment force of insects. It can be even more reduced by detachment of wax from the leaf surface by insects, since wax was found on insects pads (Gorb et al., 2005). In addition, specific wax compounds can play a role in the resistance or susceptibility to insect attacks. It was shown, that a higher amount of primary alcohols, in some cultivars of *Brassica oleracea*, reduces the attachment of the beetle *Hippodamia convergens* (Eigenbrode and Jetter, 2002). It seems, that a major function of wax in plant-insect interactions is to build a defense barrier, however, there is a possibility that they may serve as defense compounds, so-called phytoalexins too. Nonetheless, this function is only speculative. There is no research concerning wax and insect interaction in Arabidopsis. The availability of comprehensive mutant collections and the knowledge about wax biosynthesis might help to understand the mechanisms of this interaction on the biological as well as on the chemical level. However, this knowledge transfer might cause difficulties due to a lack of a model insect for feeding studies in Arabidopsis.

1.5.1.2 Jasmonoyl-isoleucine – the major wound hormone

As mentioned before, insect attacks are initiating wound signaling pathways. The main phytohormone which orchestrates the wound signaling is JA and more precisely its amino acid conjugate jasmonoyl-isoleucine (JA-Ile) (Wasternack, 2007). JA is present in all flowering plants. It plays a crucial role not only in the wound response but also for plant reproduction, as mutants being deficient in the biosynthesis of this phytohormone are sterile (Feys et al., 1994; von Malek et al., 2002). Biosynthesis of JA may start with a release of α-18:3 from the inner plastidial envelope and its oxidation by specific lipoxygenases (Figure 7) (Feussner et al., 1995; Creelman and Mullet, 1997; Delker et al., 2006).

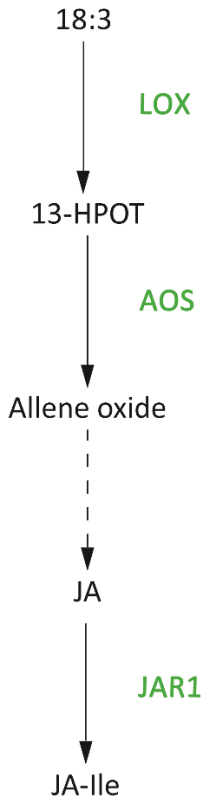

Figure 7. Simplified pathway of jasmonoyl-isoleucine biosynthesis.
α-18:3 is oxygenized to form (13*S*,9*Z*,11*E*,15*Z*)-13-hydroperoxy-9,11,15-octadecatrienoic acid (13-HPOT) by lipoxygenase (LOX), which is further catalyzed to allene oxide by allene oxide synthase (AOS). Mutants lacking this enzyme are commonly named *dde2-2* or *aos.* Through other reactions jasmonic acid (JA) is formed and it is conjugated to isoleucine by the JA-amido synthetase (JASMONIC ACID RESISTANCE 1, JAR1).

A crucial enzyme from this pathway is the ALLENE OXIDE SYNTHASE (AOS). Mutant plants lacking this enzyme, commonly named *dde2-2* or *aos,* are completely deficient in JA (von Malek et al., 2002). The active form of JA, JA-Ile, is obtained via its conjugation to isoleucine by the jasmonic acid-amido synthetase, called JASMONIC ACID RESISTANCE1 (JAR1) (Staswick et al., 2002; Westfall et al., 2012). The amount of JA-Ile increases rapidly after wounding in locally wounded leaves (Bruckhoff et al., 2016) but also in systemic leaves (Koo et al., 2009). JA-Ile binds to a co-receptor complex composed of JASMONATE ZIM domain (JAZ) and CORONATINE INSENSITIVE1 (COI1), which is the F-box protein component of the E3 ubiquitin ligase, SCF^{COI1}. This interaction triggers the degradation of JAZ and de-repression of MYC transcription factors that initiate JA dependent expression of defense-related genes (Wasternack and Hause, 2013; Goossens et al.,

2016; Zhang et al., 2017). Metabolites, which synthesis pathways are activated via JA signaling are e.g. anthocyanins, antioxidants against ROS or glucosinolates, that play a role in plant defense (Brader et al., 2001; Mikkelsen et al., 2003; Chen et al., 2006). The importance of JA signaling in the plant defense was proven by the use of mutant plants impaired in JA biosynthesis or perception. Arabidopsis mutants lacking 18:3, cannot defend themselves sufficiently against larvae of the fungal gnat, *Bradysia impatien*s (McConn et al., 1997), whereas tomato mutants impaired in JA biosynthesis are susceptible towards tissue-chewing *Manduca sexta, Spodopteraexigua* larvae, cell-content feeding *Tetranychus urticae* and the westernflower thrips *Frankliniella occidentalis* (Howe et al., 1996; Li et al., 2002; Thaler et al., 2002). Arabidopsis mutants of COI1 are more susceptible for necrotrophic fungi like *Alternaria brassicicola* and *Botrytis cinerea* but also towards *Pseudomonas syringae* (Thomma et al., 1998; Rossi et al., 2011). Moreover, caterpillars of the species *Pieris rapae* and aphids of the species *Myzus persicae* fed more efficiently on those plants (Ellis and Turner, 2002; Reymond et al., 2004). However, resistance towards many pathogens does not require the presence of JA signaling. Arabidopsis mutants lacking components of this pathway are resistant to the fungus *Leptosphaeria maculans* and to the oomycete *Phytophthora porri* (Roetschi et al., 2001; Bohman et al., 2004). Furthermore, a wound-induced JA independent phosphorylation pathway was described, showing that JA is not the only key player in the wound signaling (Rojo et al., 1998).

1.5.1.3 Regeneration process upon wounding – sealing the wounded area

Upon wounding stress, plants start to adjust their metabolism for the production of defense compounds as well as building blocks for tissue regeneration. One of the first regeneration processes is sealing the wounded area to prevent further damages e.g. by fungal or bacterial infection, but also to prevent water loss, which might cause drought stress. A well-known component involved in sealing the wounding site is callus. Callus is an undifferentiated mass of unorganized parenchymal cells. Its growth at the wounded site is promoted by cytokines (Ikeuchi et al., 2019). However, it is negatively regulated by JA, since mutants of JA biosynthesis and perception, are accumulating more callus after wounding (Ikeuchi et al., 2017). Interestingly, it

was found that the lack of one of the FAE components for the formation of VLCFA - KCS1 - results in enhanced callus formation too (Shang et al., 2016) (more about VLCFA elongation see chapter 1.4.1.1). Also the polysaccharide callose, that is associated with the cell wall, is produced to possibly seal the damaged area (Jacobs et al., 2003). Callose is deposited not only in response to wounding but also during desiccation or pathogen attack (Brown Jr et al., 1996; Chen and Kim, 2009). Moreover, another plant hormone, salicylic acid can induce its deposition (Wang et al., 2013). As mentioned previously, suberin, a FA-based polyester is known to be synthesized in response to wounding (Kolattukudy, 2001). Suberin is structurally similar to cutin, however it contains more primary alcohols, whose biosynthesis is also triggered by wounding (Domergue et al., 2010). For Arabidopsis no further data exist suggesting other metabolites, which may seal the wounding site.

1.5.2 Plant response to drought

Drought is one of the most common and one of the biggest restrictions for proper growth and development of plants. Plants growing in conditions of reduced water availability are adjusting their roots growth and elongation and reducing the growth of their green tissues. Drought triggers biosynthesis of ABA, which is causing stomatal closure to minimize water loss by transpiration and induces expression of genes involved in drought stress response (Lim et al., 2015). ABA also plays an important role in seed germination and development (Bentsink and Koornneef, 2008). ABA biosynthesis is activated by Ca^{2+} accumulating in the cytosol during drought (Figure 8). It starts from the epoxidation of zeaxanthin in plastids. After several biosynthetic steps, ABA aldehyde is oxidized to ABA by abscisic aldehyde oxidase (AAO). When levels of ABA are increasing, ABA binds to the receptor complex PYRABACTIN RESISTANCE1 (PYR)/PYR1-like (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR1 (RCAR) and to the 2C-TYPE PROTEIN PHOSPHATASE (PP2C) repressor. This leads to the activation of SNF1-RELATED PROTEIN KINASE 2 (SnRK2) and subsequently to the phosphorylation and activation of many transcription factors involved in drought stress response (Nakashima and Yamaguchi-Shinozaki, 2013; Cui et al., 2016). It was shown that the ABA signaling pathway is involved in the regulation of wax biosynthesis, since mutants of components of the ABA pathway exhibit an impaired cuticle phenotype (Cui et

al., 2016). Mutants of genes involved in ABA biosynthesis in tomato showed a reduced wax content and cuticle thickness (Martin et al., 2017). During long-term drought stress, the wax load

Figure 8. Simplified pathway of abscisic acid biosynthesis.
Increased concentration of Ca^{2+}, cause by drought leads to ABA biosynthesis. The first step is the formation of zeaxanthin, from which by further oxidations ABA-aldehyde is formed. The last step of ABA biosynthesis, the oxidation from ABA aldehyde is catalyzed by abscisic aldehyde oxidase (AAO).

is increasing by 80 % and external application of ABA is strongly inducing wax biosynthesis (Kosma et al., 2009; Seo et al., 2011). However, ABA did not induce the biosynthesis of primary alcohols, a component of wax (Kosma et al., 2009). Moreover, many transcription factors regulating genes from the wax pathway are dependent on ABA (Figure 3). That hints to ABA as the major regulator of wax biosynthesis not only under normal conditions, but upon drought stress as well. Taken together, land plants developed strategies to protect themselves from drought by increasing the

wax content on their leaf surface. This strategy limits the non-stomatal water loss and ensures survival under conditions of reduced water availability.

1.5.3 TAG accumulation in leaves upon stress – an universal stress response

During normal growth conditions, TAG is primarily accumulating in developing seeds. However, its induced biosynthesis was also observed in vegetative tissues, like leaves. The main function of TAG may be storage of FA, which later can serve as a source for energy or as building blocks for anabolism e.g. during germination or the regeneration of tissues. It was shown that TAG accumulates in spinach upon ozone treatment whereas polar lipids like PC or galactolipids were reduced (Sakaki et al., 1994). Further experiments revealed that the TAG level is increasing during freezing stress. It was found that *SENSITIVE TO FREEZING* (*SFR2*), coding for a glycosyl hydrolase of family 1, plays an important role in the cold acclimatization by removing galactosyl moieties from MGDG and forming DAG, which is further acylated to TAG (Moellering et al., 2010). This is also supported by the fact that the main accumulating FA in TAG are polyunsaturated, which are usually found in lipids from plastidial membranes. This degradation of MDDG from plastidial membranes might lead to shrinking of chloroplasts, which takes place during freezing or desiccation stresses (Moellering et al., 2010; Moellering and Benning, 2011). Induced TAG biosynthesis is also happening during senescence. This process seems to be correlated with plastid degradation during plants' aging. Upon senescence, the main accumulating FA in TAG are again polyunsaturated and this accumulation could be even increased by overexpression of the transcription factor LEC2 (Kaup et al., 2002; Lippold et al., 2012). The same phenomenon was found to take place upon wounding. Comprehensive lipidome analyses performed by liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) revealed that TAG is accumulating in response to wounding. While the total amount of MGDG and DGDG is reduced upon wounding, TAG species consisting of polyunsaturated FA (PUFA) (18:3 and 16:3) are strongly enriched (Vu et al., 2014; Vu et al., 2015). Most recently, a comprehensive study comparing the lipidome and transcriptome of heat-treated plants showed that TAG are accumulating also under these stress conditions and that membrane lipids consisting of PUFA are degraded. It was

suggested, that minimizing the amount of PUFA containing lipid species in the membranes might help the plant to adjust to elevated temperatures. The incorporation of PUFA in the TAG pool serves as its storage (Higashi et al., 2015). It seems that TAG accumulation in Arabidopsis leaves is a universal stress response, which occurs upon ozone treatment, freezing stress, wounding, senescence and heat stress as a consequence of membrane degradation and remodeling.

1.6 Aims of the study

Neutral lipids such as wax or TAG are essential for plants. Regulation of their biosynthesis have been studied for years in normal as well as in stress conditions. Despite that, there are still missing parts of this regulation, which need to be found. The main goal of this thesis was to further unravel the regulation of wax biosynthesis and TAG biosynthesis, especially upon wounding.

The second chapter of the thesis is focusing on wax and TAG biosynthesis upon wounding. The function of those two lipids upon this stress condition remained unknown as well as its regulation. Wax and TAG biosynthesis is controlled by ABA, however the wound response is orchestrated by JA-Ile. Hence, the aim of the research included in the second chapter was to reveal the function of wax and TAG biosynthesis upon wounding and to investigate the role of the two main stress hormones - ABA and JA-Ile in those processes.

The third chapter is concentrated on unravelling the role of the MYB41 transcription factor in wax biosynthesis. Its role in this process was enigmatic, although it was known that MYB41 might induce wax biosynthesis, however it also plays a role as repressor of the response to salinity. Aim of this part was to bring closer the role of MYB41 in wax biosynthesis by generating and using a collection of mutants of this transcription factor obtained via a CRISPR/Cas9 approach.

Taking all together, the overall aim of this thesis is to further resolve the regulation of wax and TAG biosynthesis and additionally their functions upon wounding.

Chapter 2: Wounding triggers wax and TAG biosynthesis in *A. thaliana* leaves.

The article is ready for submission. The supplemental materials are attached at the end of the chapter.

Author contribution

Milena Lewandowska designed all experiments. She performed expression studies via qRT-PCR, wax analysis, TAG analysis by GC-FID as well as by LC-MS/MS and membrane lipid analysis by LC-MS/MS. She analyzed the data, displayed and interpreted the results and wrote the first version of the manuscript.

Wounding triggers wax and triacylglycerol biosynthesis in Arabidopsis leaves

Milena Lewandowska[1], Krzysztof Zienkiewicz[1], Agnieszka Zienkiewicz[1], Kirstin Feussner[1,2], Ljerka Kunst[3] and Ivo Feussner[1,2,4]*

[1]*Univeristy of Goettingen, Albrecht-von-Haller-Institute for Plant Sciences, Department of Plant Biochemistry, Goettingen, Germany.*

[2]*Univeristy of Goettingen, Goettingen Center for Molecular Biosciences (GZMB), Service Unit for Metabolomics and Lipidomics, Goettingen, Germany.*

[3]*University of British Columbia, Department of Botany, Vancouver, Canada.*

[4]*Univeristy of Goettingen, Goettingen Center for Molecular Biosciences (GZMB), Department of Plant Biochemistry, Goettingen, Germany.*

*Correspondence: Ivo Feussner, Tel: +49-551-395743, E-mail: ifeussn@unigoettingen.de, ORCID iD: 0000-0002-9888-7003

Summary

- Wounding caused by insects or abiotic factors such as wind and hail is a severe stress for plants. We investigated if wax and triacylglycerol (TAG) contribute to adaptation processes after mechanical wounding, and how wax and TAG accumulation are regulated in Arabidopsis.
- The expression of genes involved in wax and TAG biosynthesis was analyzed in jasmonic acid (JA)-deficient (*dde-2*), abscisic acid (ABA)-reduced (*aao3-4*), and *myb96-1* mutants. The wax, TAG, and plant hormone content was determined. Moreover, wounding experiments in high humidity conditions were conducted to dissect wounding and drought stress responses.
- After wounding, surface wax and TAG biosynthesis is induced in wild type plants on transcript and metabolite levels, but only wax accumulation is dependent on JA, ABA, and MYB96. Plant growth and wounding under high humidity conditions prevent abscisic acid formation and the up-regulation of wax biosynthesis.
- ABA and JA are involved in regulating wound-induced wax biosynthesis, and MYB96 is a key regulator of this process; however, they are not involved in wound-induced TAG accumulation in Arabidopsis. We provide a comprehensive model of this process.

Key Words: Drought stress, lipid metabolism, mechanical wounding, phytohormone, plant defense

INTRODUCTION

Plants are exposed to many environmental stresses, one of which is mechanical wounding caused by wind, hail, or insects. Wounding leads to cell disruption, production of reactive oxygen species (ROS), and eventually to cell death (Orozco-Cardenas & Ryan, 1999; Mittler *et al.*, 2011). The membranes and walls of damaged cells lose their integrity, which leads to massive metabolic shifts. The surrounding cells seal the wound site to protect the remaining tissue from further damage, pathogen attack, or water loss, using callus (Ikeuchi *et al.*, 2017), callose (Jacobs *et al.*, 2003), or lipids such as suberin (Kolattukudy, 2001; Domergue *et al.*, 2010). These processes are often accompanied by degradation of organelles in wounded cells and deposition of fatty acids released from disrupted membranes in triacylglycerol (TAG) (Kaup *et al.*, 2002; Troncoso-Ponce *et al.*, 2013; Vu *et al.*, 2014). Subsequently, cells surrounding the wound site differentiate and proliferate to regenerate the damaged tissue (Iwase *et al.*, 2011; Sugimoto *et al.*, 2011).

The wound response is orchestrated by the active form of jasmonic acid (JA) - jasmonoyl-isoleucine (JA-Ile), which regulates the expression of defense genes (Farmer & Ryan, 1990; Farmer *et al.*, 2014; Wasternack & Feussner, 2018). The amount of JA-Ile increases rapidly after wounding (Koo *et al.*, 2009; Bruckhoff *et al.*, 2016; Zhang *et al.*, 2016). JA-Ile binds to a co-receptor complex composed of JASMONATE ZIM domain (JAZ) and an E3 ubiquitin ligase complex CORONATINE INSENSITIVE1 (COI1). This interaction triggers the degradation of JAZ and the de-repression of MYC transcription factors that initiate JA-dependent expression of defense-related genes (Wasternack & Hause, 2013; Goossens *et al.*, 2016; Zhang *et al.*, 2017). Mutants deficient in the precursors of JA, α-linolenic acid and roughanic acid, cannot mount a sufficient defense against insects (McConn *et al.*, 1997) and mutants of *COI1* are more susceptible to necrotrophic fungi (Thomma *et al.*, 1998; Rossi *et al.*, 2011). Moreover, plants treated with JA or methyl-jasmonate (MeJA) exhibit a greater defense response against herbivores and necrotrophs (Thomma *et al.*, 1998; Kessler & Baldwin, 2001). Another mutant of the JA biosynthesis pathway with disrupted *ALLENE OXIDE SYNTHASE* (*AOS*), accumulates more callus around the wound site than wild type (WT) plants (Ikeuchi *et al.*, 2017). In addition, mutants of this enzyme exhibit a male-sterile phenotype, which can be rescued by MeJA application (Park *et al.*, 2002; von Malek *et al.*, 2002). Wounding performed on the

mutant of this gene, *dde2-2,* showed that on the transcriptome and metabolome level, a substantial part of the wound response is JA-independent (Kaever *et al.*, 2015).

Another plant hormone synthesized in response to wounding is abscisic acid (ABA) (Bostock & Stermer, 1989; Pena-Cortes *et al.*, 1995). It plays a major role in the response to abiotic stresses such as drought or osmotic stress (Cutler *et al.*, 2010). When levels of ABA increase, the ABA receptor complex PYRABACTIN RESISTANCE1 (PYR)/PYR1-like (PYL)/REGULATORY COMPONENT OF ABA RECEPTOR1 (RCAR) binds to ABA and to 2C-TYPE PROTEIN PHOSPHATASE (PP2C) repressor. This leads to activation of SNF1-RELATED PROTEIN KINASE2 (SnRK2), phosphorylation, and activation of many transcription factors involved in the stress response (Nakashima & Yamaguchi-Shinozaki, 2013; Cui *et al.*, 2016). One of the ABA-dependent and stress-activated transcription factors is MYB96. It is involved in defense against pathogens (Seo & Park, 2010) and in the drought response (Seo *et al.*, 2009; Lee *et al.*, 2016). Additionally, MYB96 together with MYB94 additively regulate wax biosynthesis (Seo *et al.*, 2011; Lee *et al.*, 2016). Wax covers all primary aerial surfaces of plants, and in *A. thaliana*, it is primarily composed of very long chain fatty acids (VLCFA) and their derivatives such as alkanes, aldehydes, alcohols, ketones and wax esters, which typically range from 24 to 34 carbons in length. MYB94 and MYB96 are involved in the first step of wax biosynthesis, VLCFA elongation, by regulating the expression of *ECERIFERUM2* and *ECERIFERUM6 (CER2* and *CER6). CER6* encodes β-KETOACYL-CoA SYNTHASE6, which catalyzes the first reaction of elongation and is the rate-limiting enzyme (Millar & Kunst, 1997; Fiebig *et al.*, 2000; Hooker *et al.*, 2002). *CER2* encodes a protein of unknown function involved in elongation of VLCFA (Haslam *et al.*, 2012). In addition, MYB94 and MYB96 regulate the expression of genes involved in the alkane-forming pathway, *ECERIFERUM1 and ECERIFERUM 3* (*CER3*), and the alcohol-forming pathway, *WAX SYNTHASE/ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE1* (*WSD1*) (Lee *et al.*, 2016). The expression of two genes encoding wax transporters, *LIPID TRANSFER PROTEIN G2* (*LTPG2)* and *ATP BINDING CASSETTE G11* (*ABCG11)*, is also controlled by MYB96 (Seo *et al.*, 2011). Recently it was shown that this transcription factor directly regulates TAG accumulation by binding to the *PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1* (*PDAT1*) promotor, and indirectly by regulating *ACYL-CoA:DIACYLGLYCEROL ACYLTRANSFERASE1* (*DGAT1*) expression (Lee *et al.*,

2018). Both of these genes encode acyltransferases that catalyze the last step of TAG biosynthesis (Katavic *et al.*, 1995; Zou *et al.*, 1999; Dahlqvist *et al.*, 2000).

A comprehensive metabolome and transcriptome analysis of wounded WT and JA-deficient mutant (*dde2-2*) plants showed that many genes involved in wax (Fig. 1) and TAG biosynthesis (Fig. 2) are expressed independently of JA-Ile (Kaever *et al.*, 2015). Genes involved in wax precursor elongation (*CER6, CER2, CER10*), alkane formation (*CER1, CER3*), and wax transport (*LTPG2*) were induced at least slightly within 2 hours post wounding (hpw), mainly independently of JA (Fig. 1). A similar expression pattern was observed for *DGAT1,* however, expression of genes encoding the enzymes involved in the early steps of TAG biosynthesis (Fig. 2), including *ACYL-CoA:GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE9* (*GPAT9*), *ACYL-CoA:LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE2* and *3* (*LPAAT2* and *LPAAT3*), *PHOSPHATIDIC ACID HYDROLASE1* and *2* (*PAH1* and *PAH2*), and *ACYL-PDAT1* were not induced within 2 hpw.

The mechanisms regulating wax and TAG biosynthesis upon wounding in flowering plants have remained unknown. In this study, we show that wax accumulates upon wounding, that this process requires elevated levels of both JA-Ile and ABA, and that MYB96 is a key regulator. In turn, we show that TAG biosynthesis in response to wounding is JA-Ile, ABA, and MYB96-independent.

MATERIALS AND METHODS

Plant materials and growth conditions

The following *A. thaliana* lines were used in this study: Columbia-0 ecotype, *dde2-2* (von Malek *et al.*, 2002), *aao3-4* (kindly provided by Prof. Dr. Christiane Gatz, University of Goettingen), *myb96-1* (Seo *et al.*, 2009), *myb94-1* (Lee & Suh, 2014) (both kindly provided by Prof. Dr. Mi Chung Suh, Chonnam National University), *dgat1-1* and *pdat1-2* (kindly provided by Prof. Dr. John Ohlrogge, Michigan State University). All plants were grown in growth chambers under following conditions, unless specified otherwise: white light illumination (130-150 µmol m^{-2} s^{-1}) under short day conditions (8 h light : 16 h dark) at 22 °C during the day and at 18 °C at night, with c. 60 % humidity. For experiments with inflorescence stems, plants were grown under long day conditions (16 h light : 8 h dark). For high humidity experiments the plants were grown in trays tightly sealed with plastic lids and covered with plastic foil. 5 days before

wounding, the plants were transferred to a chamber with c. 90 % humidity at night and 96 % humidity during the day. Plants were grown on soil supplemented 4:1 with vermiculite.

Stress treatment

Rosette leaves of 7-8-week-old plants were mechanically wounded using forceps (Stenzel *et al.*, 2003). Inflorescence stems of 4-5 week-old plants were wounded every 0.5 cm from the bottom up to the first siliques. For gene expression profiling, phytohormone measurements, and TAG and membrane lipid analysis, completely wounded rosettes or inflorescence stems were harvested and immediately frozen in liquid N_2. For each experiment, six to ten plants were pooled at each time point. For expression and phytohormone measurements plants were harvested at 0, 0.5, 2, and 5 hours post wounding (hpw), with the exception of high humidity experiments where time point 0.5 hpw was skipped. For TAG and membrane lipid analysis plants were harvested at 0 and 24 hpw, whereas fresh material for wax analysis was harvested at 0 and 6 hpw. For MeJA treatment, plants were sprayed with 5 mM MeJA in 0.1 % aqueous Tween20 solution 24 h before wounding.

Determination of phytohormones by UPLC-nano ESI-MS/MS

Extraction of 100 mg of plant material was preceded with 0.75 ml methanol containing 10 ng of each of the following internal standards: D6-SA, D6,ABA, D5-JA (all three from C/D/N Isotopes Inc., Pointe-Claire, Canada), and D4-JA-Leu (kindly provided by Otto Miersch, Halle/Saale, Germany). Afterwards the sample was vortexed and 2.5 ml of methyl-tert-butyl ether (MTBE) was added. The sample was shaken for 1 h at 4 °C and next 0.6 ml water was added for phase separation. The extract was incubated at room temperature for 10 min and centrifuged at 450 x *g* for 15 min. The upper phase was collected and to the lower phase 1.3 ml MTBE and 0.7 ml methanol/water (3:2.5, v/v) was added and the sample re-extracted as described previously. The upper phases were combined and dried under N_2 stream. Afterwards the extract was resuspended in 100 µl of acetonitrile/water (20:80, v:v) containing 0.3 mmol l^{-1} NH_4HCOO (adjusted to pH 3.5 with formic acid).

The reverse phase separation was carried out with a UPLC using an ACQUITY UPLC® system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 column (100 mm x 1 mm, 1.8 µm; Waters Corp., Milford, MA, USA). Injection was

performed in partial loop with needle overfill mode and the elution of sampled was adapted as described (Balcke *et al.*, 2012). Solvents used were water and acetonitrile/water (90:10, v/v) (solvent A and B, respectively) and both were supplemented with 0.3 mmol l^{-1} NH_4HCOO (adjusted to pH 3.5 with formic acid). The separation temperature was 40 °C and the flow rate was set to 0.16 ml min^{-1}. The gradient program was performed isocratically for 0.5 min at 10 % of solution B, followed by a linear increase to 40 % of solution B in 1.5 min. This condition was kept for 2 min, followed by a linear increase to 95 % of solution B in 1 min, and then held for 2.5 min. The starting conditions of the column were achieved by re-equilibration in 3 min.

Nanoelectrospray ionization (nanoESI) analysis was performed using a chip ion source (TriVersa Nanomate®; Advion BioSciences, Ithaca, NY, USA). For stable nanoESI, 70 µl min^{-1} of 2-propanol/acetonitrile/water (70:20:10, v:v:v) containing 0.3 mmol l^{-1} NH_4HCOO (adjusted to pH 3.5 with formic acid) delivered by a Pharmacia 2248 HPLC pump (GE Healthcare, Munich, Germany) were added via a mixing tee valve just after the column. The eluent was directed by using another post column splitter, 502 nl min^{-1}, to the nanoESI chip with 5 µm internal diameter nozzles. The ionization voltage was established to -1.7 kV. The ionization of phytohormones was performed in negative mode and measured in scheduled multiple reaction monitoring mode with an AB Sciex 4000 QTRAP® tandem mass spectrometer (AB Sciex, Framingham, MA, USA). Mass transitions were as previously described (Iven *et al.*, 2012) with some modifications, which are listed in Table 1.

Table 1 – Analyzed phytohormones with their mass transitions (Q1 and Q3) declustering potential (DP), entrance potential (EP), and collision energy (CE)

Q1 [Da]	**Q3 [Da]**	**Analyte**	**DP [V]**	**EP [V]**	**CE [V]**
137	93	SA	-25	-6	-20
141	97	D4-SA	-25	-6	-22
209	59	JA	-30	-4.5	-24
214	62	D5-JA	-35	-8.5	-24
263	153	ABA	-35	-4	-14
293	179	D6-ABA	-80	-10	-42
322	130	JA-Ile/Leu	-45	-5	-28
325	133	D3-JA-Leu	-80	-4	-30

The mass analyzers were adjusted to a resolution of 0.7 atomic mass units full width at half-height. The temperature of the ion source was 40 °C, and the curtain gas was set to 10 (given in arbitrary units). Quantification was performed by using a calibration curve of intensity (*m/z*) ratios of [unlabeled]/[deuterium-labeled] *vs.* molar amounts of unlabeled (0.3-1000 pmol).

Analysis of transcript levels

qRT-PCR and microarray analysis were used to investigate gene expression levels. Total RNA was extracted using TRI-reagent (Sigma-Aldrich) according to the manufacturer's protocol. RNA quantification was carried out with a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). 1 µg of RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA was synthesized using Revert Aid H Minus Reverse Transcriptase (Thermo Fisher Scientific). qRT-PCR was performed using Takyon No ROX SYBR Mastermix blue dTTP (Kaneka Eurogentec) in a 20 µl reaction volume. The gene *ACTIN8* was used as a reference. All primers used in this experiment are listed in Supplemental Table 1. Each reaction was performed with material from plants harvested in three independent experiments in an iQ5 real time detection system (Bio-Rad). Microarray analysis was performed with an Agilent-021169 Arabidopsis 4 Oligo Microarray (V4) platform. Expression values were quantile-normalized and spots without gene assignment were removed (Kaever *et al.*, 2015).

Wax extraction and analysis

Wax was extracted from leaves of 6-8-week-old plants and from stems of 5-6-week-old plants. Stems or leaves were immersed in chloroform containing tetracosane (Sigma-Aldrich) as an internal standard. Samples were then dried under an N_2 stream and re-dissolved in 10 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and 10 µl of pyridine (Sigma-Aldrich). Derivatization was performed at 80 °C for 1 h. Afterwards samples were dried under an N_2 stream and re-dissolved in 10 µl of chloroform. Wax was quantified by gas chromatography with a flame ionization detector (Agilent GC 6890, Agilent Technologies) coupled with a 30 m HP-1 column using helium as a carrier gas. 2 µl of each sample was injected with a 1:5 split for leaf samples and 1:15 for stem samples. Gas chromatography was carried out with the oven temperature set to 50 °C for 2 min then raised by 40 °C min^{-1} to 200 °C and then

held for 1 min, afterwards raised by 3 °C min^{-1} up to 320 °C and then held for 15 min. The signals were integrated using the ChemStation Software (Agilent Technologies). Wax compounds were identified beforehand with gas chromatography linked with mass spectrometric detector (Agilent 5973 Network, Agilent Technologies). Quantification of wax amount was determined by comparing the peak areas from ionization detector to the internal standard. Leaves and stem areas were determined as described (Haslam & Kunst, 2013).

Confocal microscopy

For lipid droplet visualization, leaves from Arabidopsis plants were cut into small pieces and then fixed with 4 % (v:v) paraformaldehyde in 0.01 M phosphate-buffered saline (PBS), pH 7.0, for 30 min at room temperature. Next, samples were rinsed with PBS and stained with 10 µg ml^{-1} BODIPY^{TM}505/515 (ThermoFisher Scientific) for 1 h at room temperature. After two washes with PBS the samples were analyzed with a TSC-SP5 Laser Scanning Confocal Microscope (Leica Microsystems, Wetzlar, Germany). BODIPY was excited using an argon laser (ex. 488 nm) and emission was detected and captured at 510-530 nm. Chlorophyll autofluorescence was excited with the same laser at 594 nm and emission was observed and captured at 620-640 nm. Images were merged using ImageJ.

Aniline blue staining was used to visualize callose deposition after wounding. Whole leaves of Arabidopsis were destained for 24 h in 1:3 (v:v) acetic acid : ethanol. Transparent leaves were washed in 150 mM K_2HPO_4 for 30 min and afterwards incubated for 2 h in 150 mM K_2HPO_4 and 0.01 % aniline blue. Samples were embedded in 50 % glycerol for analysis. Tissues were examined with a Meta Confocal Laser Scanning Microscope (Carl Zeiss Mikroskopie; Jena, Germany) with a diode laser exciting at 405 nm.

Extraction and analysis of triacylglycerols (TAG) by TLC and GC-FID

Lipids from pulverized plant material (500 mg) were extracted with 1 volume of 2:1 (v:v) chloroform:methanol and shaken for 1 h. Prior to extraction, tri-15:0 TAG (Sigma-Aldrich) was added as an internal standard. Afterwards, 0.5 volume of 1 M KCl 0.2 M H_3PO_4 solution was added (Dörmann *et al.*, 1995). The nonpolar phase was collected and dried under an N_2 stream, then dissolved in chloroform and separated by thin layer chromatography (TLC) using hexane:diethyl ether:acetic acid (80:20:1, v:v:v) on F60

silica gel glass plates (Merck). The TAG band was visualized by spraying TLC plates with 8-anilino-1-naphthalenesulfonic acid 0.2 % (w:v) and exposure to UV light. TAG bands were scraped off of silica plates and transesterified to generate fatty acid methyl esters (FAMEs). Afterwards FAMEs were quantified by gas chromatography with a flame ionization detector (Agilent GC 6890, Agilent Technologies) as described (Zulu *et al.*, 2017).

Extraction and analysis of triacylglycerols (TAG) and plastidial lipids by UPLC-nano ESI-MS/MS

For LC-MS/MS analysis, lipids were extracted with 2-propanol:hexane:water (60:26:14) (v:v:v) with incubation at 60 °C for 30 min. The extract was centrifuged and the supernatant was collected and split evenly in two aliquots. Both aliquots were evaporated to dryness under an N_2 stream, and then reconstituted in 400 µl tetrahydrofuran/methanol/water, 4:4:1 or 7:2:1 (v:v:v, for membrane lipids and TAG, respectively). The analysis was carried out with a UPLC using an ACQUITY UPLC® system (Waters Corp., Milford, MA, USA) equipped with an ACQUITY UPLC® HSS T3 column (100 mm x 1 mm, 1.8 µm; Waters Corp., Milford, MA, USA) coupled with MS/MS system (QTRAP 6500®, AB Sciex, Framingham, MA, USA) as described previously (Tarazona *et al.*, 2015). Samples were injected at a volume of 2 µl with the needle in overfill mode, a flow rate of 0.1 ml min^{-1}, and a separation temperature of 35 °C.

Statistical analysis

Data were statistically analyzed by a one-way ANOVA with Tukey's post-hoc test using R studio v.1.1456.

RESULTS

Wounding leads to a strong JA-Ile-independent accumulation of transcripts related to wax and TAG biosynthesis

JA-Ile orchestrates the plant´s response to wounding (Howe *et al.*, 2018). In order to dissect jasmonate-dependent and -independent responses, the jasmonate-deficient *dde2-2* mutant was used. The expression patterns revealed by microarray analysis

(Figures 1 and 2) were confirmed by qRT-PCR analyses. The genes involved in wax and TAG biosynthesis are up regulated in response to wounding mainly in a JA-Ile-independent manner (Fig. 3 a-j, light vs. dark grey bars). Genes encoding enzymes of the initial steps of wax biosynthesis, *CER2, CER6*, and *CER10*, are induced in WT plants within 5 hpw c. 4 to 6-fold. However, *CER6*, which encodes the main condensing enzyme associated with wax metabolism, was not significantly induced in jasmonate-deficient plants. In contrast, genes encoding enzymes and transporters involved in the later steps of wax biosynthesis, such as *CER1* and *LTPG2,* showed much higher expression in *dde2-2*. Interestingly, these genes showed 100- and 60-fold accumulation rates, respectively, in *dde2-2* leaves 5 hpw. In WT those rates were 30 for *CER1* and 20 for *LTPG2* (Fig. 3e and 3h, light vs. dark grey bars).

Transcripts for the enzymes catalyzing the last step of TAG assembly, *DGAT1* and *PDAT1*, are up regulated after wounding in a JA-Ile-independent manner. The expression of *DGAT1* was induced up to 70-fold and *PDAT1* up to 4-fold in both WT and *dde2-2* (Fig. 3i-j, light vs. dark grey bars).

TAG and wax accumulate after wounding

After transcript analysis, wax and TAG content was compared between wounded and non-wounded plants. The total TAG content in leaves increased within 24 hpw from 50 to 170 µg/g fresh weight in both WT and in *dde2-2* plants (Fig. 4a, light vs. dark grey bars). The polyunsaturated fatty acids 16:3, 18:2, and 18:3 contributed most to this increase (Fig. S1, light vs. dark grey bars). These results are in agreement with our qRT-PCR data, where a high accumulation of both *DGAT1* and *PDAT1* transcripts was observed after wounding. By comparison, the increase in wax accumulation after wounding was much subtler. Measurement of wax content by GC-FID showed that c. 40 % more wax content could be detected on WT leaves 6 hpw in comparison to non-wounded leaves (Fig. 4b, light vs. dark grey bars). Significant changes in alkane and fatty acid content were observed 6 hpw (Fig. S2, light vs. dark grey bars). Though wax content was the same for non-wounded WT and *dde2-2*, surprisingly, wax did not accumulate after wounding in the JA-Ile-deficient mutant. This observation is in contrast to transcript profiles, as expression of most genes involved in wax biosynthesis was significantly induced in *dde2-2* after wounding (Fig. 3a-h, light vs. dark grey bars). In fact, some wax-related genes such as *CER1, LTPG2* (Fig. 3e, h,

light vs. dark grey bars), and the wax biosynthesis regulator *MYB96* (Fig. S3) were even more strongly up regulated in *dde2-2* plants in comparison to WT. There is no published evidence of a connection between JA-Ile and leaf cuticular wax content. To examine the effect of JA-Ile on wax accumulation in response to wounding, we attempted to recover the WT response to wounding in *dde2-2* plants with MeJA treatment. MeJA was sprayed on WT and *dde2-2* mutant plants 24 h before the wounding experiment. MeJA application increased wax content on the non-wounded WT plants by c. 7% in comparison to the plants growing without of MeJA-treatment (Fig. 4 vs. S4), but did not influence the wound-induced wax content in WT (Fig. 4 vs. S4). Interestingly, for the JA-Ile-deficient mutant wax accumulation was similar to that of WT plants after both MeJA application and wounding. These findings strongly suggest that the presence of JA-Ile is essential for wax accumulation after wounding.

Plants deficient in JA-Ile accumulate Salicylic acid (SA) and callose upon wounding

We decided to asses, how the absence of JA-Ile influences the wax accumulation after wounding. It is known, that JA-Ile and SA can work antagonistically (Robert-Seilaniantz *et al.*, 2011), therefore we measured the amount of SA in wounded and non-wounded plants. SA amount did not increase in WT plants up to 5 hpw, however in JA-Ile-deficient mutant it was significantly higher 5 hpw (Fig. S5a, light vs. dark grey bars). Moreover, it is known that SA induces callose deposition in Arabidopsis (Wang *et al.*, 2013) and it is well established that callose is formed after wounding (Jacobs *et al.*, 2003). Thus, we analyzed also the callose deposition in *dde2-2* and WT plants after wounding (Fig. S5b). A higher number of callose plugs were observed in the wounded JA-Ile-deficient plants in contrast to the wounded WT plants. This observation let us hypothesize that the amounts of wax was not enriched in *dde2-2* plants after wounding, because sealing the wounding site by callose is the more promoted process when SA is enriched in absence of JA-Ile.

Wax accumulation is impaired in *aao3-4* and *myb96-1*, but TAG accumulation is not

It was recently shown that both wax and TAG biosynthesis are regulated by the same MYB96 transcription factor (Seo *et al.*, 2011; Lee *et al.*, 2018). MYB96 expression is controlled by the plant drought hormone ABA (Seo *et al.*, 2009). Therefore, a knockout

mutant of MYB96 *(myb96-1)* and a mutant with reduced ABA (*aao3-4*) were analyzed to test if ABA signaling plays a role in wax biosynthesis upon wounding. In non-wounded *aao3-4* plants, the amount of ABA was reduced by 70 % in comparison to non-wounded WT. However, ABA still accumulated significantly in wounded *aao3-4* plants between 2 and 5 hpw (Fig. S6b, light grey vs. blue bars), to c. 26 % of the amount observed in wounded WT plants 5 hpw. In order to investigate if ABA and MYB96 regulate expression of genes related to wax biosynthesis after wounding, qRT-PCR analysis was performed. In wounded leaves of *aao3-4*, only a slight induction was observed for *CER6, CER1, CER3*, and for the transporter *ABCG11* (Fig. 3a, e, f, g, blue bars). However, a significant induction was shown for *CER2, CER10,* and *LTPG2* (Fig. 3b, c, h, blue bars). These results correlate well with the reduced ABA accumulation in *aao3-4* after wounding (Fig. S6b, blue bars), and suggest that the increased accumulation of transcripts associated with wax metabolism in response to wounding is partially ABA-dependent. The wax load of *aao3-4* leaves was constitutively reduced by c. 40% compared to WT (Fig. 4b, blue bars). This decrease was most noticeable for alkanes (Fig. S2, blue bars). The wax load increased by c. 30% after wounding, but it was less pronounced than in WT (Fig. 4b, light grey vs. blue bars). These data confirm that ABA is an important wound response factor.

It was previously shown that *myb96-1* accumulates less wax on leaves and stems than WT, although the expression of genes involved in wax biosynthesis were not changed in *myb96-1* plants under normal growth conditions (Seo *et al.*, 2011). We confirmed these findings and showed that after wounding none of the wax synthesis-related genes are significantly up regulated in *myb96-1* plants (Fig. 3a-h, yellow bars). The constitutive wax load of *myb96-1* was reduced by 40% relative to WT (Fig. 4b, yellow bars), and the greatest decrease was observed for alkanes (Fig. S2, yellow bars). Moreover, in *myb96-1* wax accumulation upon wounding did not take place. From these results we conclude that MYB96 is a key regulator of wax accumulation in the wound response.

In contrast to wax biosynthesis genes, two genes governing the final steps of TAG biosynthesis, *DGAT1* and *PDAT1,* were induced upon wounding in *aao3-4* and WT plants (Fig. 3i-j, blue bars). In *myb96-1* plants, a slight reduction of *DGAT1* transcript accumulation was observed after wounding when compared to WT (Fig. 3i, yellow bars). In contrast to that, *PDAT1* did not accumulate in *myb96-1* after wounding (Fig. 3j, yellow bars). These results are in accordance with a previously reported direct

regulation of *PDAT1* expression by MYB96 and its indirect control on *DGAT1* expression (Lee *et al.*, 2018). Similarly, TAG accumulation in *aao3-4* and *myb96*-1 plants was observed (Fig. 4a, yellow and blue bars), although the total content reached only 70 % of that in WT plants before and after wounding.

High humidity prevents wound-induced wax accumulation in Arabidopsis leaves

Taking into consideration that ABA is a main drought stress hormone and that it is also involved in wound-induced wax accumulation (Fig. 4b, light grey vs. blue bars), clearly distinguishing between drought stress and wound stress responses is necessary. Arabidopsis plants were grown and wounded at high humidity (c. 96 %). First, the expression of genes involved in wax biosynthesis before and after wounding was analyzed. None of the tested genes were up regulated after wounding in high humidity (Fig. 5a), although the plants responded to the stress treatment since elevated JA-Ile was observed at 0.5 and 2 hpw (Fig. 5b). Interestingly, the concentration of JA-Ile was c. 10-fold lower in wounded plants grown in high humidity than in wounded plants grown in normal humidity conditions (Fig. S6a and Fig. 5b). In contrast to JA-Ile, there was no significant increase in ABA amount after wounding (Fig. 5c). Upon wounding in normal humidity conditions ABA amount increases 2.5-fold up to 5 hpw (Fig. S6b), whereas in high humidity conditions this increase was c. 1.8 fold 5hpw, nevertheless this difference is not significant. Next, we assessed the wax content of Arabidopsis leaves grown and wounded in high humidity. A strong reduction (c. 48%) of wax load was observed in non-wounded leaves in comparison to plants grown in normal humidity conditions (Fig. 4d and Fig. 5d). However, the wax content did not change before and after wounding in high humidity (Fig. 5d). These results imply that an elevated amount of ABA, as a consequence of wound-induced drought stress, is necessary for enhanced wax production after wounding.

Wounding of Arabidopsis inflorescence stems does not induce wax biosynthesis

Inflorescence stems of Arabidopsis are covered by c. 40 times more wax than leaves (Fig. 4b and Fig. 6d). We investigated whether wax biosynthesis in inflorescence stems can also be stimulated by wounding. qRT-PCR-analyses were performed with wounded and non-wounded inflorescence stems of WT and JA-Ile-deficient *dde2-2* mutant plants. No significant induction of genes involved in wax biosynthesis was

observed in WT and *dde2-2* plants after wounding (Fig. 6a), with the exception of *CER2* and *LTPG2*, which showed higher transcript accumulation. As expected, the JA-Ile-dependent wound response was active in the inflorescence stems immediately after wounding, since JA-Ile rapidly increased in WT, but not in the *dde2-2* mutant plants (Fig. 6b). Surprisingly, no induction of ABA was observed in either plant line (Fig. 6c), although it is known that ABA can be produced and transported in inflorescence stems (Hartung *et al.*, 2002; Koiwai *et al.*, 2004). Wax amount was measured 4, 6, and 8 hpw in inflorescence stems (Fig. 6d), with an additional sample (R) harvested just after wounding. This sample was analyzed to exclude mechanical abrasion of wax during the wounding procedure. Changes in wax content after wounding were not detected in WT and JA-Ile-deficient plants. This observation is consistent with the hypothesis that drought stress-induced ABA accumulation is essential for the induction of wax biosynthesis upon wounding. It seems that the response of stems to wounding, unlike leaves, does not involved ABA or wax.

TAG composed of polyunsaturated fatty acids accumulates in and around wound sites

Arabidopsis leaves have elevated TAG content after wounding (Vu *et al.*, 2014; Vu *et al.*, 2015). We showed that this process is JA-Ile- and ABA-independent (Fig. 3i, j, Fig. 4a). Confocal microscopy showed that TAG is enriched in lipid droplets at and around the wounding site (Fig. 7), while these organelles were very rare in unwounded Arabidopsis leaves under our experimental conditions (Fig. S7). As a control, the lipid droplet formation was also analyzed in plants 1 hpw, however the abundance of those round-shaped organelles was not detected at this time point. (Fig. S8). Moreover, expression of a gene coding the main lipid droplet-associated protein in leaves, *CALEOSIN3,* was increased up to 40-fold in WT plants 2 hpw (Fig. S9) (Naested *et al.*, 2000). We aimed to assess which of the acyltransferases catalyzing TAG formation is responsible for inducing TAG content upon wounding. Knockout mutants of two main genes required for the last step of TAG assembly, *DGAT1* or *PDAT1,* accumulate TAG upon wounding, however in reduced amount in comparison to WT (Fig. 4a, pink and green bars).

The TAG accumulation in WT, *dde2-2, aao3-4, myb96-1* was confirmed by UPLC-MS/MS analysis (Fig. S10 and S11), where TAG species with 9 and 8 double bonds in total accumulated strongly. The highest accumulating TAG species were: 52:8 and

52:9 (Fig. 8b-c). Since polyunsaturated fatty acids, especially 16:3, are mainly present in plastidial membranes (Ohlrogge & Browse, 1995), the abundance of the major species of the plastidial lipid classes monogalactosyldiacylglycerol (MGDG), digalactosyldiacylglycerol (DGDG), and phosphatidylglycerol (PG) was also analyzed. In parallel to the strong accumulation of TAG in all tested lines 24 hpw, a slight decrease of the amount of plastidial lipids was observed (Fig. 8a-b, Fig. S10-S17). The main species of MGDG, DGDG (34:6 composed of 16:3 and 18:3; 36:6 composed of two times 18:3 (Fig. S13 and S15)), and PG (34:4 composed of 16:1 and 18:3; 34:3 composed of combinations of 16:1, 16:0, 18:2 and 18:3 (Fig. S17-S18)) were present in reduced amounts in wounded leaves.

Taking all these observations together, we found that TAG accumulate at and around the wounding site and that they are mainly composed of polyunsaturated fatty acids. TAG accumulation was observed in all tested lines: the JA-Ile-deficient mutant, ABA-educed mutant, *myb96-1*, and even *dgat1-1* and *pdat1-2*. This indicates that the process of TAG biosynthesis after wounding is not dependent on the two major wounding hormones JA-Ile and ABA. Even mutants of two main TAG-producing enzymes *pdat1-2* and *dgat1-1* are able to accumulate TAG.

DISCUSSION

Previous work showed that plants can use different materials to seal wound sites, including callose, callus, and suberin (Kolattukudy, 2001; Jacobs *et al.*, 2003; Domergue *et al.*, 2010; Ikeuchi *et al.*, 2017). In this study we demonstrated that surface wax is also produced in response to wounding. Moreover, we analyzed the regulation of wax and TAG accumulation after wounding by using mutants impaired in the biosynthesis of two major plant hormones, JA-Ile and ABA. Mutants of two major transcription factors involved in wax biosynthesis, *MYB96* and *MYB94*, were also included in the analysis. Finally, we demonstrated that a second class of neutral lipids, TAG, is synthesized in response to wounding, and that this process seems to be independent of the two main wounding hormones, ABA and JA-Ile.

Wax biosynthesis upon wounding is JA-Ile- and ABA-dependent and MYB96 is a major regulator of this process

Wax is synthesized from saturated fatty acids composed of 16 or 18 carbons in length. Firstly, these are elongated by a Fatty Acid Elongation (FAE) complex, which includes CER6, CER2, CER10, and several other enzymes (Samuels *et al.*, 2008; Haslam *et al.*, 2012). Afterwards the pathway splits into two: an alkane-forming pathway and an alcohol-forming pathway. In the alkane-forming pathway, two enzymes, CER1 and CER3, play major roles in producing alkanes and aldehydes (Bernard *et al.*, 2012). In the alcohol-forming pathway, VLCFA are reduced to alcohols by a Fatty Acid Reductase (FAR) (Rowland & Domergue, 2012). Fatty alcohols are esterified with C16-C18 fatty acids to form wax esters by enzymes from the WSD family (Li *et al.*, 2008). The produced wax components can then be transported across the plasma membrane and cell wall to the plant surface by ABC and LTPG transporters, respectively (Pighin *et al.*, 2004; Bird *et al.*, 2007; DeBono *et al.*, 2009). We analyzed the expression of genes coding for enzymes of all steps of the wax biosynthesis pathway and determined the wax content in WT, *dde2-2*, *aao3-4*, and *myb96-1* after wounding (Fig. 3 and Fig. 4b). All genes tested were up regulated after wounding independently of JA-Ile, except for *CER6.* Furthermore, some of the genes involved in wax accumulation including *CER1*, *LTPG2*, and *MYB96* had substantially higher expression in the JA-Ile-deficient *dde2-2* than in WT after wounding. Surprisingly, in the JA-Ile-deficient line, wax accumulation after wounding was not observed, in contrast to WT plants, and in conflict with most of the gene expression patterns we observed. JA-Ile could regulate wax biosynthesis by transcriptional activation of *CER6*, however, as expression of this key enzyme required for wax precursor elongation was not induced upon wounding of the *dde2-2* mutant. Furthermore, some of the genes involved in wax biosynthesis like *CER1* or *LTPG2*, but also *MYB96* have much higher expression in *dde2-2* than in WT after wounding, probably to additionally reinforce the pathway whereas its products are not formed. The lack of wax accumulation after wounding is related to the absence of JA-Ile, since we could restore wax accumulation completely by external application of MeJA (Fig. S4). Until now, there were no data suggesting a relation between wax biosynthesis and JA-Ile. In JA-Ile-deficient plants, callus formation and callose deposition after wounding was much more pronounced than in WT plants, as was shown previously (Ikeuchi *et al.*, 2017) (Fig. S5b). Perhaps these different materials serve similar and interchangeable functions in sealing the wound site.

To date, ABA was the only plant hormone known to play a role in wax biosynthesis. Increased ABA activates many transcription factors such as MYB94 or MYB96 (Broun

et al., 2004; Lee & Suh, 2013). It was previously shown that ABA amount increases after wounding, which we confirmed in our studies (Fig. 6b). We analyzed the wound response in the ABA-reduced mutant *aao3-4* and showed that these plants still exhibit ABA response to wounding, since its amount increases c. 2.1 times, but more weakly than WT plants. Since *AAO3* is not the only ABA oxidase in Arabidopsis, it is not surprising that the *aao3* mutant has a subtle phenotype (Seo *et al.*, 2000). Expression analyses of *aao3-4* plants showed that only some of the genes involved in wax biosynthesis are up regulated after wounding (Fig. 3). Similar results were obtained for ABA-signaling mutants in Arabidopsis, for example mutants of SNF1-Related Protein Kinase 2, where expression of genes involved in wax biosynthesis was reduced (Cui *et al.*, 2016). In previous research on ABA-reduced lines of tomato, expression of *CER3, ABCG11*, and *CER6* genes decreased and the plants accumulated less wax (Martin *et al.*, 2017). As expected, in *A. thaliana* *aao3-4* plants a similar effect was observed; the wax load was reduced in non-wounded plants in comparison to WT (Fig. 4b). After wounding, the increase in wax production was reduced in *aao3-4* relative to the induction observed in the WT as well. We concluded that elevated ABA is necessary for inducing wax biosynthesis after wounding. These results suggested that effectors downstream from ABA, such as MYB94 and MYB96, might influence wax accumulation after wounding. MYB94 and MYB96 work additively in activating wax biosynthesis in response to drought stress, in an ABA-dependent manner (Lee *et al.*, 2016). In *myb96-1* mutants, expression of none of the genes involved in wax biosynthesis was induced after wounding, indicating that MYB96 is involved in wax biosynthesis after wounding. In line with this, we did not observe any wax accumulation in these plants. Unlike for *myb96*, in the *myb94* mutant we observed a similar wound response as in WT plants (Fig. S18), suggesting that this transcription factor is not directly involved in the wound response.

Wax biosynthesis is drought stress-dependent

Since MYB96 is a transcription factor involved in wax biosynthesis in response to drought, and ABA is a main drought response factor, it was necessary to dissect the wound response from the drought stress response. Wounding of Arabidopsis leaves in high humidity showed that neither expression of genes governing wax biosynthesis nor wax accumulation increased upon wounding (Fig. 5). Further, under these

conditions the level of ABA after wounding remained unchanged. Similar results have been reported; plants wounded in high humidity did not produced more ABA upon wounding, but produced much more ROS than plants wounded in normal humidity conditions (L'Haridon *et al.*, 2011). Plants wounded in high humidity also showed higher cuticular permeability than control plants wounded under normal humidity conditions (L'Haridon *et al.*, 2011). This suggests that in high humidity, plants do not seal wound sites as effectively, or as quickly, since the risk of water loss is less under these conditions. Previous studies established that wax accumulates during drought stress in WT Arabidopsis leaves (Seo *et al.*, 2011) as well as in *myb96-1* plants, however in reduced amounts. Furthermore, it was recently shown that wax esters, which are relatively minor components of Arabidopsis cuticular wax, accumulate in response to drought stress in leaves and stems of Arabidopsis (Patwari *et al.*, 2019). Our data revealed that the genes associated with drought stress from ABA signaling, *RESPONSIVE TO DESSICATION 29A* (*RD29B)* and *ABSCISIC ACID INSENSITIVE* 1 (*ABI1)*, were strongly induced by wounding (Fig. S19). We observed that wound-induced wax biosynthesis shares similarities with drought-induced wax formation, however these processes differ with respect to the involvement of MYB94 in wound-induced response. Our results provide strong evidence that wax accumulation upon wounding is part of a drought stress response of the leaves.

Arabidopsis inflorescence stems bear 40-fold more wax than leaves. It may not be surprising that inflorescence stems and leaves grown under high humidity react similarly to wounding. Both organs showed no enhanced expression of genes coding for enzymes of wax biosynthesis and no accumulation of wax and ABA, while elevated levels of JA-Ile were detected (Fig. 6). This confirms that elevated amounts of both hormones are required for wax accumulation after wounding. Importantly, inflorescence stems of Arabidopsis contain not only more wax, but also more fibers than leaves. Fibers could make stems more resistant to the water loss associated with wounding, which would reduce the strength of the wounding response. Therefore, we proposed a model in which wounding induces part of the drought stress machinery, leading to an increase in ABA content, activation of MYB96, and wax biosynthesis to seal the wounding site (Fig. 9). However, JA-Ile is also essential to fully induce wax accumulation in response to wounding (Fig. 9)

TAG accumulates after wounding in a JA-Ile, ABA, and MYB96-independent manner

The MYB96 transcription factor regulates expression of two genes encoding enzymes that are directly involved in TAG assembly – *DGAT1* and *PDAT1.* TAG was known to accumulate after wounding, but it was unclear how this process is regulated. We found that expression of *DGAT1* and *PDAT1* is induced after wounding and that TAG accumulation is independent from JA-Ile, ABA, and *MYB96* (Fig. 3i, j). *dgat1* and *pdat1* single mutants also accumulated TAG, presumably by compensating for each other's role (Fig. 4a). Unfortunately, we cannot confirm this hypothesis, since the *dgat1pdat1* double mutant is lethal. TAG accumulates at and around the wound site, most likely at the expense of plastidial lipids from the damaged chloroplast (Fig. 7 and Fig. 8). The most abundant fatty acid in TAG accumulating after wounding is 16:3, reflecting the plastidial lipids that are reduced. Similar observations have been published previously (Vu *et al.*, 2015). TAG is most likely not synthesized *de novo* since none of the genes upstream from *DGAT1* and *PDAT1* were induced upon wounding (Fig. 2). It was proposed that PDAT1 is responsible for transferring fatty acids from membrane lipids to TAG (Fan *et al.*, 2013). In the response to wounding it does not seem to be the case, since *pdat1-2* accumulates TAG after wounding and its role can be complemented by DGAT1. It is well-established that TAG is enriched in leaves during senescence, especially TAG species with polyunsaturated fatty acids (Kaup *et al.*, 2002). Senescence is a form of programmed cell death, with metabolic shifts occurring in all tissues, and wherein chloroplasts are one of the first organelles to be degraded (Lim *et al.*, 2007). Lipids of plastidial membranes are catabolized during this process, and are incorporated into TAG (Kaup *et al.*, 2002; Troncoso-Ponce *et al.*, 2013). Presumably, TAG accumulation is a general part of a cell damage, also caused by wounding. It seems that TAG accumulation preceded by membrane lipid degradation is a universal plant stress response, since the incorporation of plastidial fatty acids was also observed in plants undergoing freezing stress (Moellering *et al.*, 2010) or ozone treatment (Sakaki *et al.*, 1990). We showed here that wounding-triggered TAG accumulation is independent of two main stress hormones, ABA and JA-Ile (Fig. 9), and that it is a result of membrane damage upon wounding.

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FIGURE LEGENDS

Fig. 1 Expression of genes involved in wax biosynthesis is wound-induced. Transcription was analyzed by DNA microarray analysis. WT and *dde2-2* were wounded across the mid-vein and harvested before (0 h), 0.5, and 2 h post wounding. Values represent means (±SE) of transcript amounts from three independent experiments*. CER – ECERIFERUM, LTPG2 – LIPID TRANSFER PROTEIN G2.*

Fig. 2 Expression of genes involved in TAG biosynthesis is wound-induced. Transcription was analyzed by DNA microarray analysis. WT and *dde2-2* were wounded across the mid-vein and harvested before (0 h), 0.5, and 2 h post wounding. Values represent means (±SE) transcript amounts from three independent experiments. GPAT – *ACYL-COA:GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE, LPAAT – ACYL-COA:LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE, PAH – PHOSPHATIDIC ACID HYDROLASE, PDAT1 - PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1), DGAT1 - ACYL-COA:DIACYLGLYCEROL ACYLTRANSFERASE1.*

Fig. 3 Expression of genes involved in wax and TAG biosynthesis is induced upon wounding. Transcript of chosen genes was analyzed by quantitative Real Time PCR (qRT-PCR) in leaves of WT, *dde2-2, aao3-4,* and *myb96-1* before (0 h), 0.5, 2, and 5 h post wounding. Relative expression of the following transcripts are shown (a-c) *CER6, CER2* and *CER10* involved in fatty acid elongation; (d) *WSD1* involved in wax ester formation; (e,f) *CER1* and *CER3* involved in alkane formation; (g,h) *ABCG11* and *LTPG2* coding wax transporters, (i,j) *DGAT1* and *PDAT1* from TAG biosynthesis. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0.05). Values represent means (±SE) of qRT- PCR analyses of plants of three independent experiments.

Fig. 4 TAG accumulates upon wounding in a JA-Ile-, ABA-, and MYB96-independent manner, whereas accumulation of surface wax is dependent on all three regulators. (a) TAG amount in leaves of WT, *dde2-2, aao3-4, myb96-1, dgat1-1*, and *pdat1-2* before (0 h) and 24 h post wounding. (b) Total wax load in Arabidopsis leaves before (0 h) and 6 h past wounding. Values are means (±SD) of GC-FID analyses of plants from three independent experiments. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0.05).

Fig. 5 High humidity prevents accumulation of ABA and wax in response to wounding in Arabidopsis leaves. (a) Expression of genes involved in wax biosynthesis in non-wounded (0 h) and wounded (2 h and 5 h) WT plants determined by qRT-PCR. None of the tested genes were significantly up regulated. Quantification of (b) JA-Ile and (c) ABA in non-wounded (0 h) and wounded (2 h and 5 h) WT plants by LC-MS/MS. (d) Total surface wax content in Arabidopsis leaves before (0 h) and 6 h post wounding. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0.05). Values represent means (±SD) of analyses of plants from three independent experiments.

Fig. 6 Wounding of Arabidopsis inflorescence stems does not trigger wax biosynthesis. (a) Expression of genes involved in wax biosynthesis before (0 h) and after wounding (2 h, 5 h) in WT and the JA-Ile-deficient mutant *dde2-2*. Transcript was analyzed by qRT-PCR. Quantification of (b) JA-Ile and (c) ABA in non-wounded (0 h) and wounded (2 h, 5 h) inflorescence stems of WT and *dde2-2* by LC-MS/MS. (d) Wax load of non-wounded (0 h), wounded and immediately extracted (R) inflorescence stems as well as stems harvested 4, 6, or 8 h post wounding were analyzed by GC-FID. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0.05). Values represent means (±SD) of analyses from three independent experiments.

Fig. 7 Lipid droplets accumulate in and around wounding sites in Arabidopsis leaves. Lipid droplet formation was analyzed by staining with BODIPY (green) in WT, d*de2-2, aao3-4, myb96-1, dgat1-1*, and *pdat1-2* lines 24 h post wounding. Arrows point to lipid droplets. Areas outlined by dotted lines and marked with W show wounded area. Analysis was performed by confocal microscopy, bars: 50 µm.

Fig. 8 TAG accumulates after wounding, while the amount of plastidial lipids is reduced. (a) Heatmap representation obtained by clustering lipid profiles by means of one-dimensional self-organizing maps. Lipid profiles of 14 TAG species, 11 DGDG species, 14 MGDG species, and 6 PG species 24 h post wounding in WT, *dde2-2, aao3-4, myb96-1* were used. (b) Clustering results on a lipid species level. (c) Boxplots showing representative lipid species from each of the two clusters, DGDG (34:5) for cluster 1 and TAG (54:9) for cluster 2, respectively. All data were normalized to non-wounded conditions for each plant line. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. 9 Proposed model for the regulation of the wax and TAG biosynthesis after wounding. JA-Ile and drought stress-induced ABA are both essential for increased wax biosynthesis upon wounding. The major regulator of this process is MYB96. Membrane damaged caused by wounding triggers TAG accumulation in an ABA- and JA-Ile-independent manner.

SUPPLEMENTAL FIGURES

Fig. S1 TAG composed of polyunsaturated fatty acids accumulates upon wounding. TAG composition after wounding was analyzed in WT, d*de2-2, aao3-4, myb96-1, dgat1-1*, and *pdat1-2*, values represent means (±SD) of GC-FID analyses of plants harvested in three independent wounding experiments.

Fig. S2 Wax alkanes, aldehydes, and fatty acids accumulate after wounding. Wax compound amounts before and 6 h after wounding. Each compound group was statistically analyzed separately. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values are means (±SD) of GC-FID analyses of plants harvested in three independent wounding experiments.

Fig. S3 Expression of *MYB96* in WT and *dde2-2* before and after wounding. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values are means (±SE) of quantitative Real Time PCR analyses of plants harvested in three independent wounding experiments

Fig. S4 Wax analysis of plants sprayed with 5 mM MeJA before (0 h) and after wounding (6 h) in WT and the JA-deficient mutant *dde2-2*. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (*P*<0,05). Values represent means (±SD) of GC-FID analyses of plants harvested in three independent wounding experiments

Fig. S5. Analysis of SA content and callose deposition. (a) SA content in WT, d*de2-2, aao3-4* and *myb96-1*. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of LC-MS/MS analysis of plants harvested at three independent wounding experiments. (b) analysis of callose formation in WT and *dde2-2* before and 24h after wounding. Analysis was performed by confocal microscopy, bars: 300 µm.

Fig. S6. Analysis of JA-Ile and ABA amount in non-wounded and wounded leaves of WT, *dde2-2, aao3-4, myb96-1* before (0 h) and 0.5, 2, 5 hours after wounding. (a), JA-Ile amount and (b), ABA amount before and after wounding. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (*P*<0,05). Values represent means (±SD) of LC-MS/MS analysis of plants harvested at three independent wounding experiments..

Fig. S7. Lipid droplets formation analyzed by BODIPY staining (green) before wounding in WT, *dde2-2, aao3-4, myb96-1*, *dgat1-1* and *pdat1-2.* Analysis was performed by confocal microscopy, bars: 50 µm.

Fig. S8. Lipid droplets formation analyzed by BODIPY staining (green) 1 hpw in WT. Analysis was performed by confocal microscopy, bars: 50 µm.

Fig. S9. Expression of *CLO3* involved in lipid droplet formation in leaves. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of GC-FID analyses of plants harvested at three independent wounding experiments.

Fig. S10. Triacylglycerol (TAG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1.* Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S11. Chosen triacylglycerol (TAG) species before and 24 h after wounding in WT, *dde2-2, aao3-4, myb96-1*. Peak area of species 52:9 is a sum of areas of 16:3 and 18:3 while 52:8 is a sum of areas of 18:2,18:3,16:2 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S12. Monogalactosyldiacylglycerol (MGDG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1.* Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S13. The most abundant monogalactosyldiacylglycerol (MGDG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Peak area of species 34:6 is a sum of areas of 16:3 and 18:3 while 36:6 is a sum of areas of 18:3 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S14. Digalactosyldiacylglycerol (DGDG) composition before and 24 h after wounding in WT*, dde2-2, aao3-4* and *myb96-1*. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S15. The most abundant digalactosyldiacylglycerol (DGDG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Peak area of species 34:6 is a sum of areas of 16:3 and 18:3 while 36:6 is a sum of areas of 18:3 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

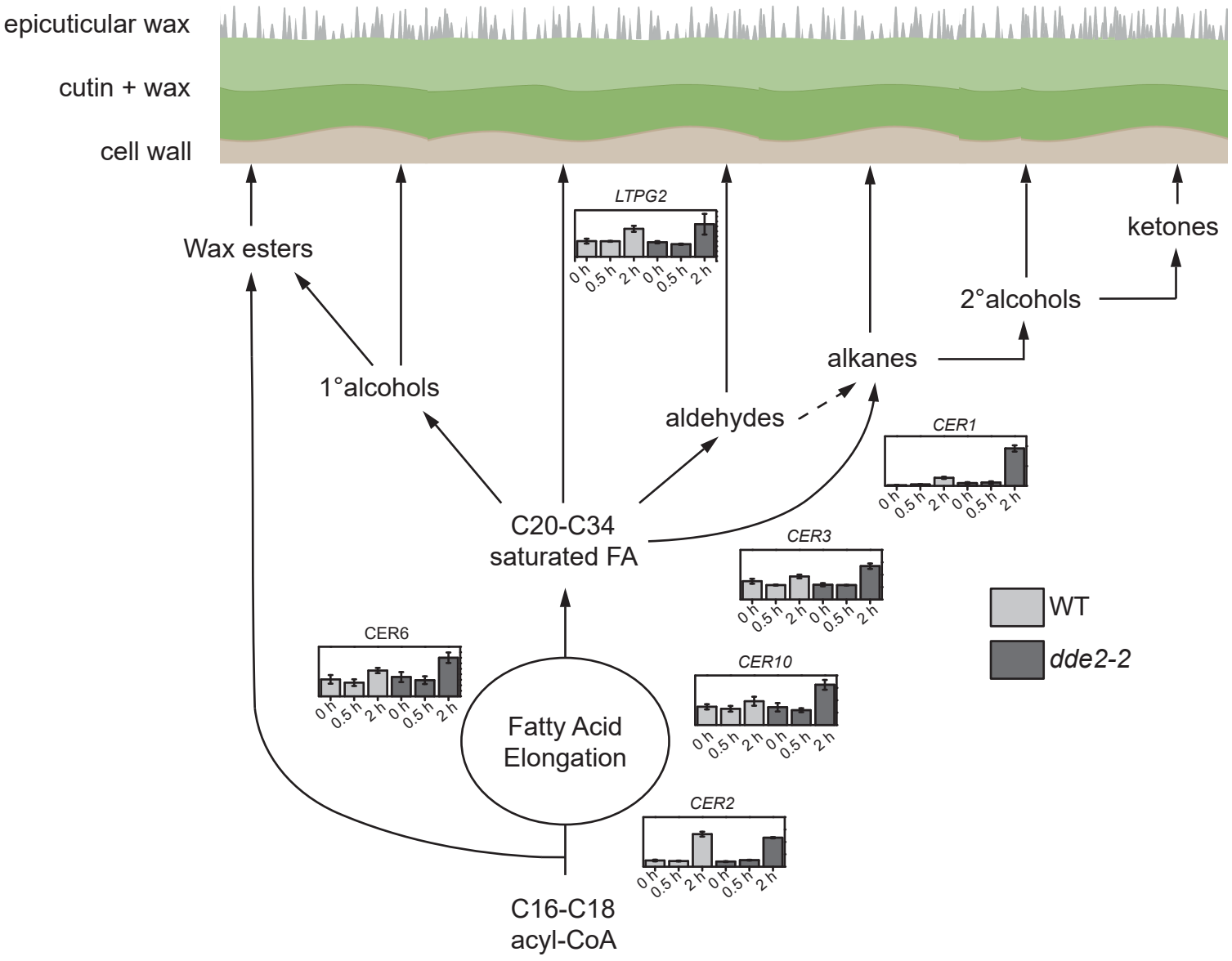
Fig. S16. Phosphatidylglycerol (PG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S17. The most abundant phosphatidylglycerol (PG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Peak area of species 34:4 is a sum of areas of 16:1 and 18:3 while 34:3 is a sum of areas of 16:1, 16:0, 18:3 and 18:2. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

Fig. S18. Response to wounding in *myb94-1.* (a) Expression of CER1 and (b) CER2 in WT and myb94-1 before (0 h) and 0.5, 2, 5 hours after wounding. Values represent means (±SE) of quantitative Real Time PCR analyses of plants harvested at three independent wounding experiments. (c), wax content before and 6h after wounding in *myb94-1.* Values represent means (±SD) of GC-FID analyses of 8 biological replicates

Fig. S19. Expression of *RD29A* and *ABI1* involved in ABA signaling in abiotic stress response. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of qRT-PCR analyses of plants harvested at three independent wounding experiments.

Table S1. Primers used in the study.



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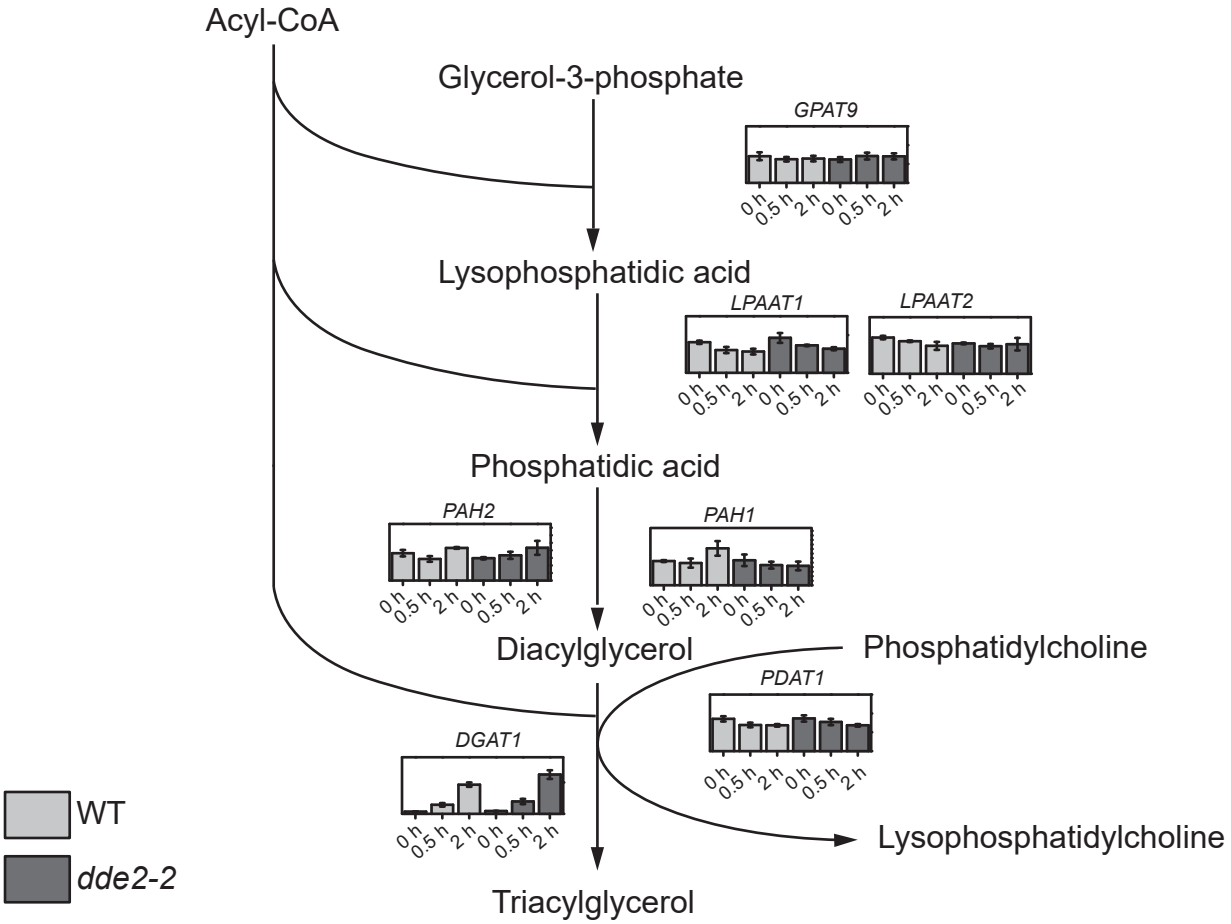
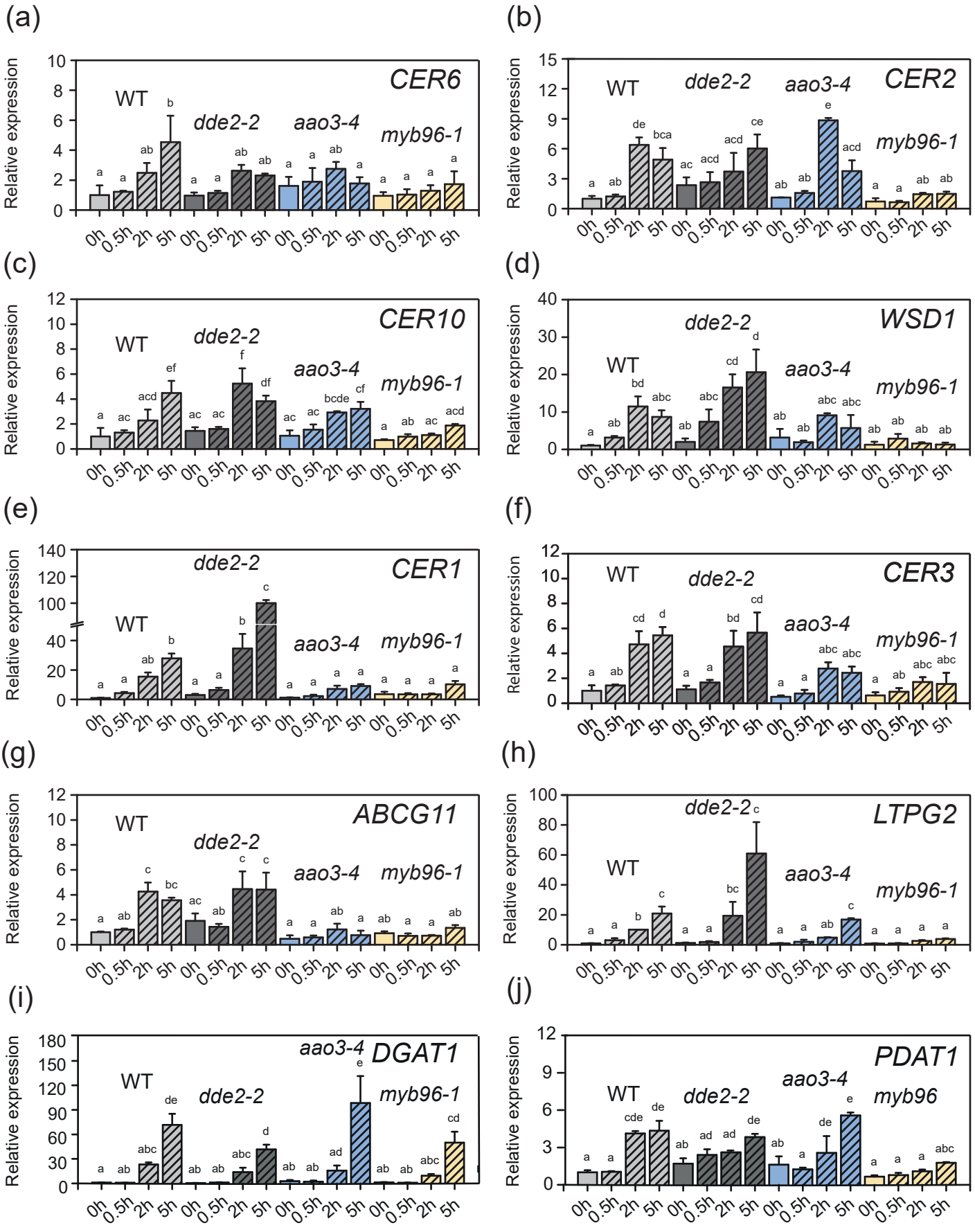


Figure 2. Expression of genes involved in TAG biosynthesis is wound-induced. Transcription was analyzed by DNA microarray analysis. WT and *dde2-2* were wounded across the mid-vein and harvested before (0 h), 0.5, and 2 h post wounding. Values represent means (±SE) transcript amounts from three independent experiments. G*PAT – ACYL-COA:GLYCEROL-3-PHOSPHATE ACYLTRANSFERASE, LPAAT – ACYL-COA:LYSOPHOSPHATIDIC ACID ACYLTRANSFERASE, PAH – PHOSPHATIDIC ACID HYDROLASE, PDAT1 - PHOSPHOLIPID:DIACYLGLYCEROL ACYLTRANSFERASE1 (PDAT1), DGAT1 - ACYL-COA:DIACYLGLYCEROL ACYLTRANSFERASE1.*



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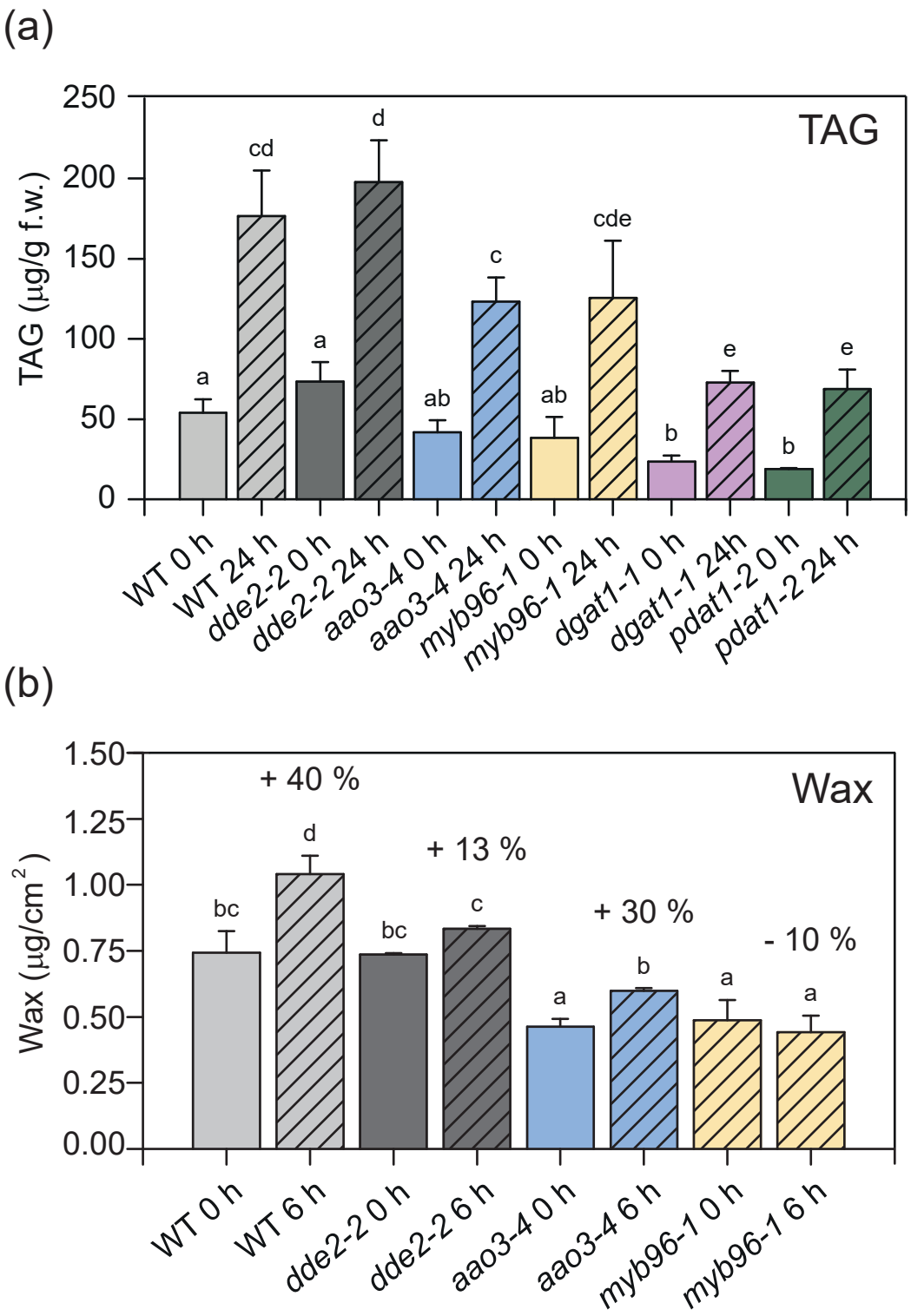

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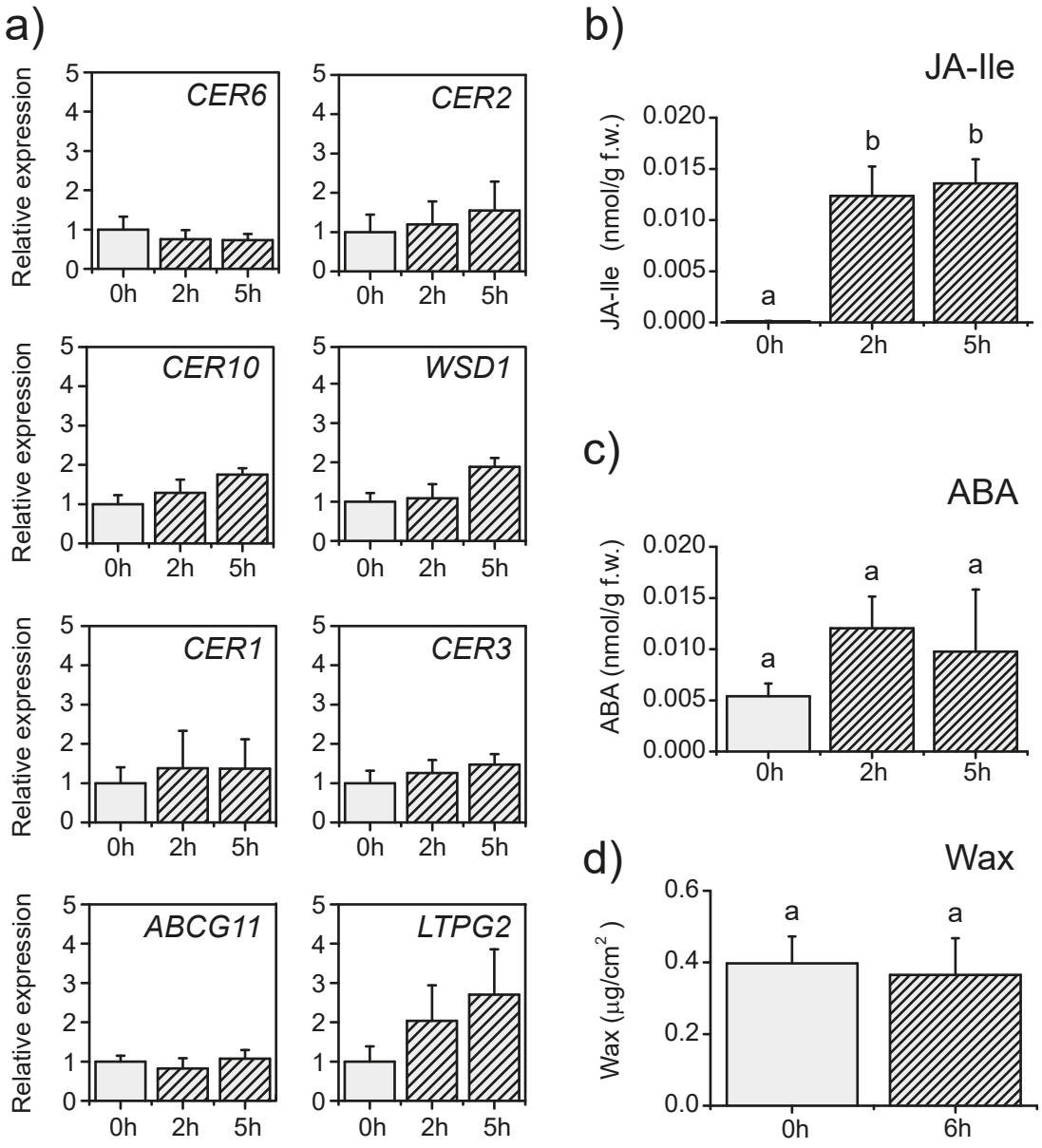

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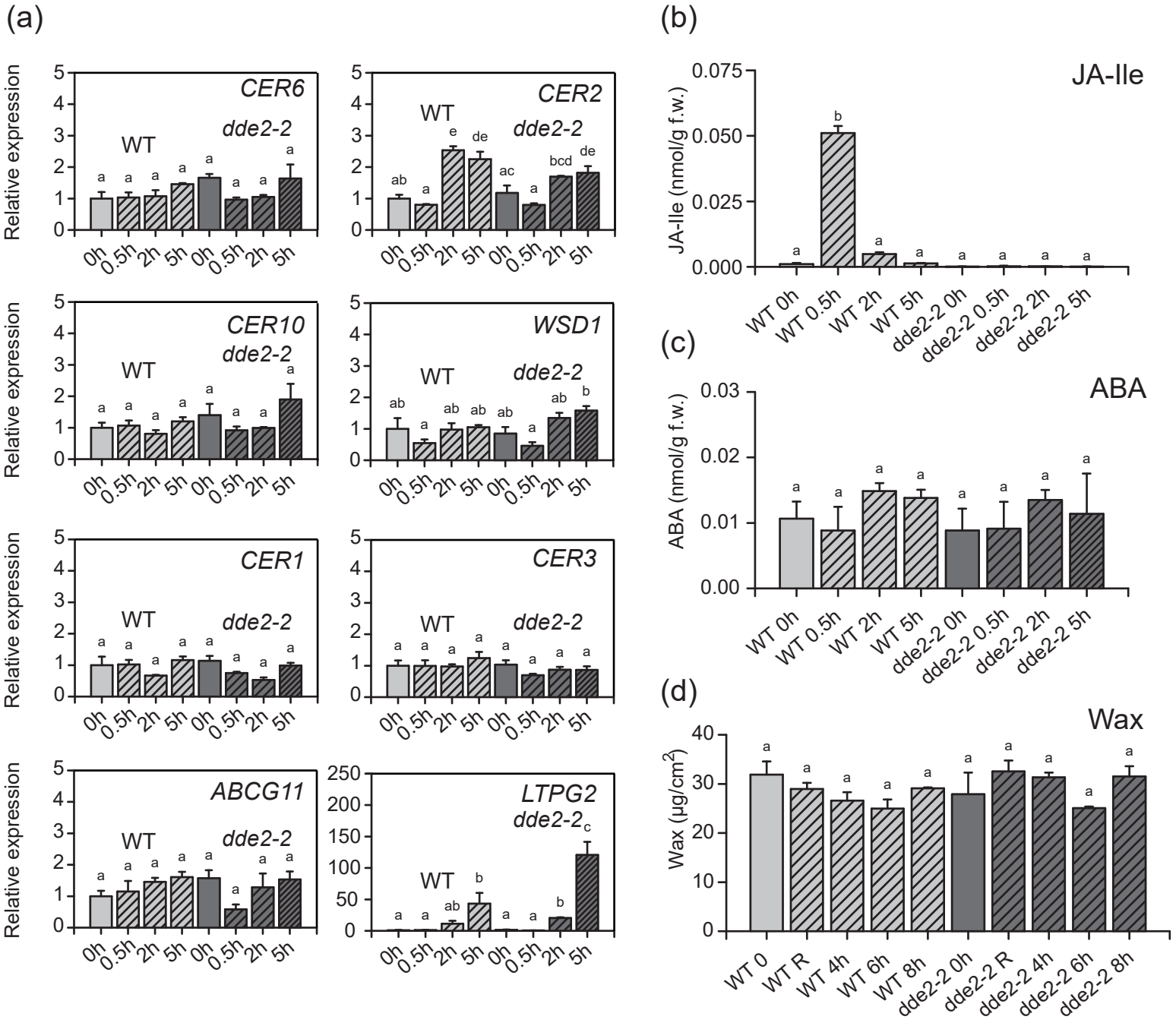

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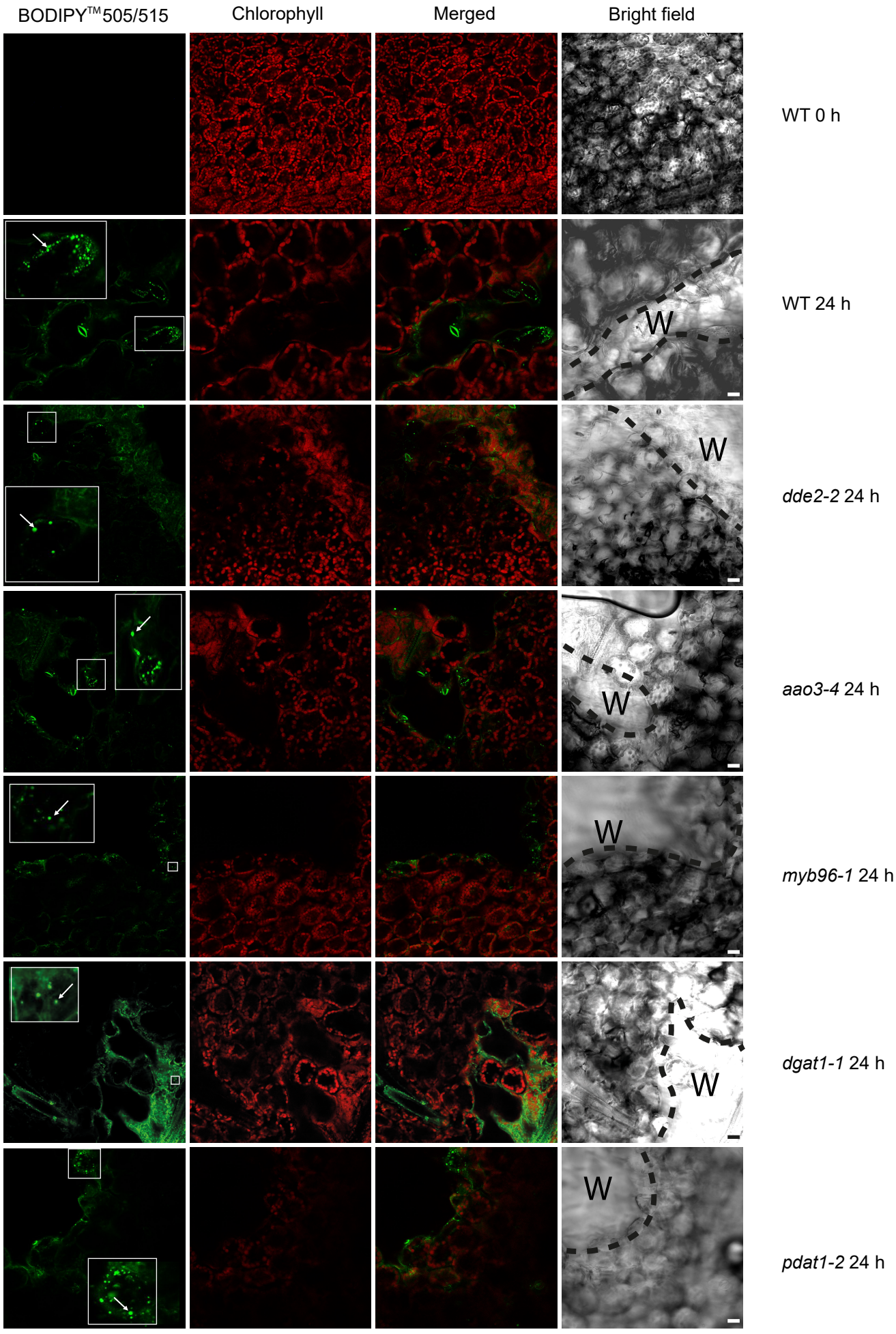

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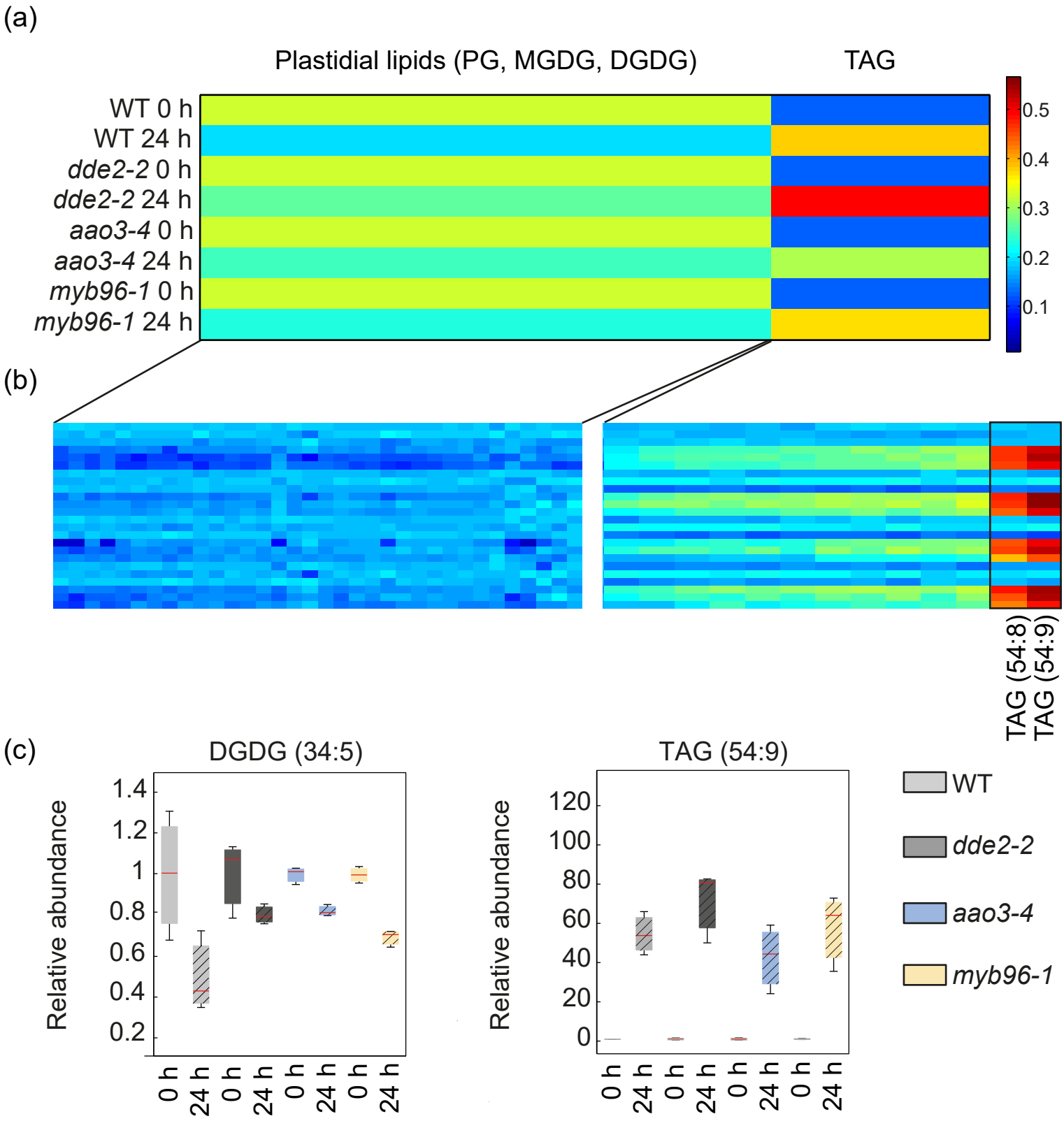

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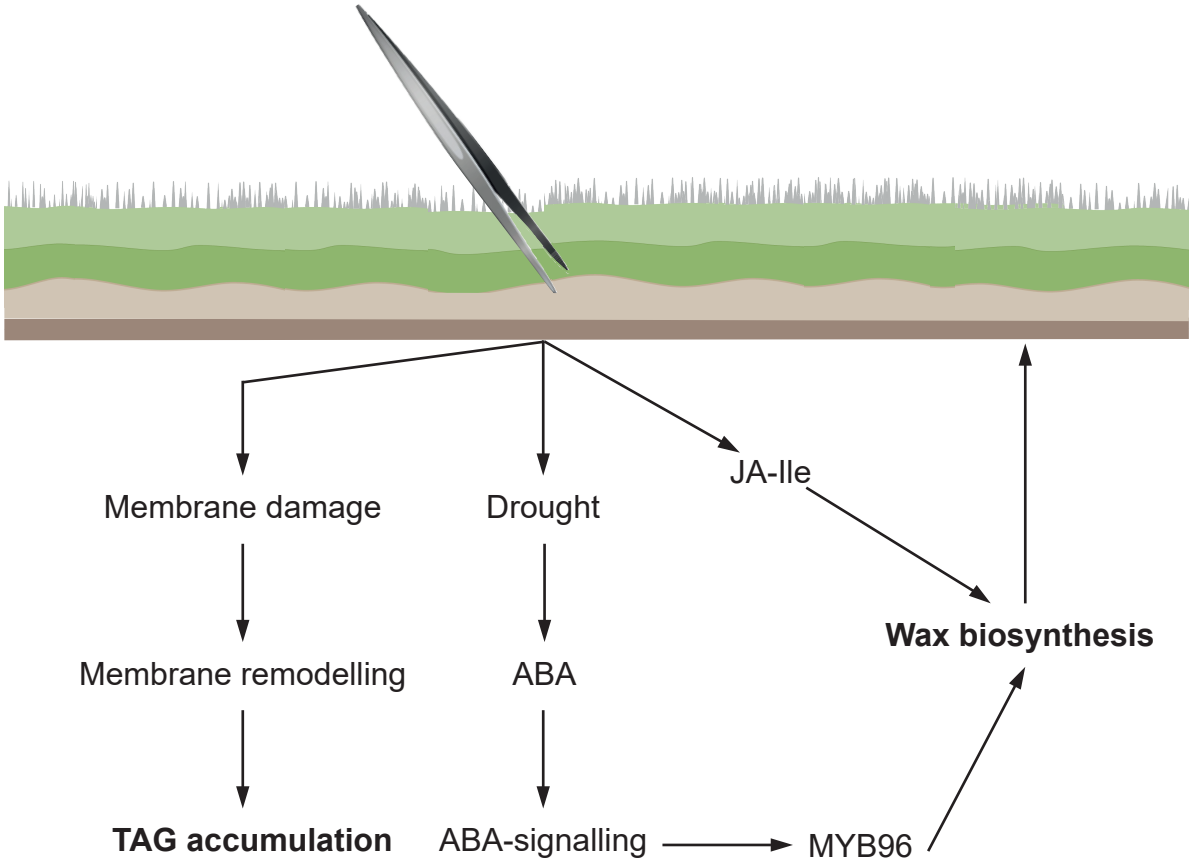

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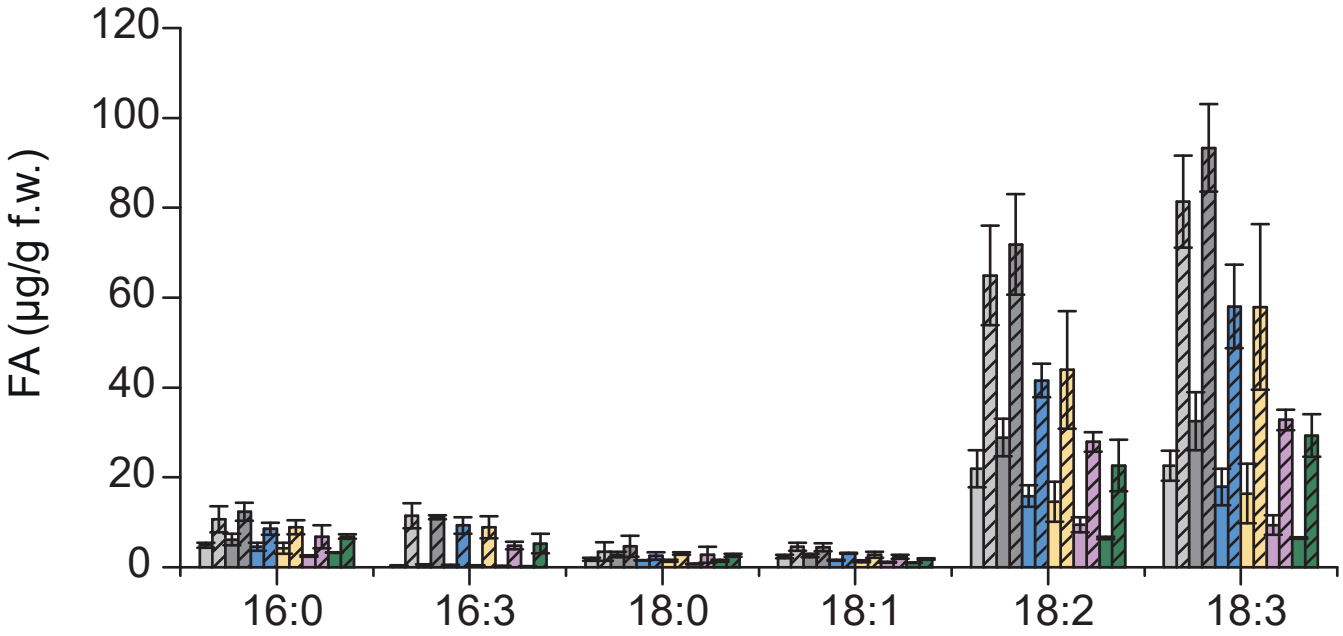

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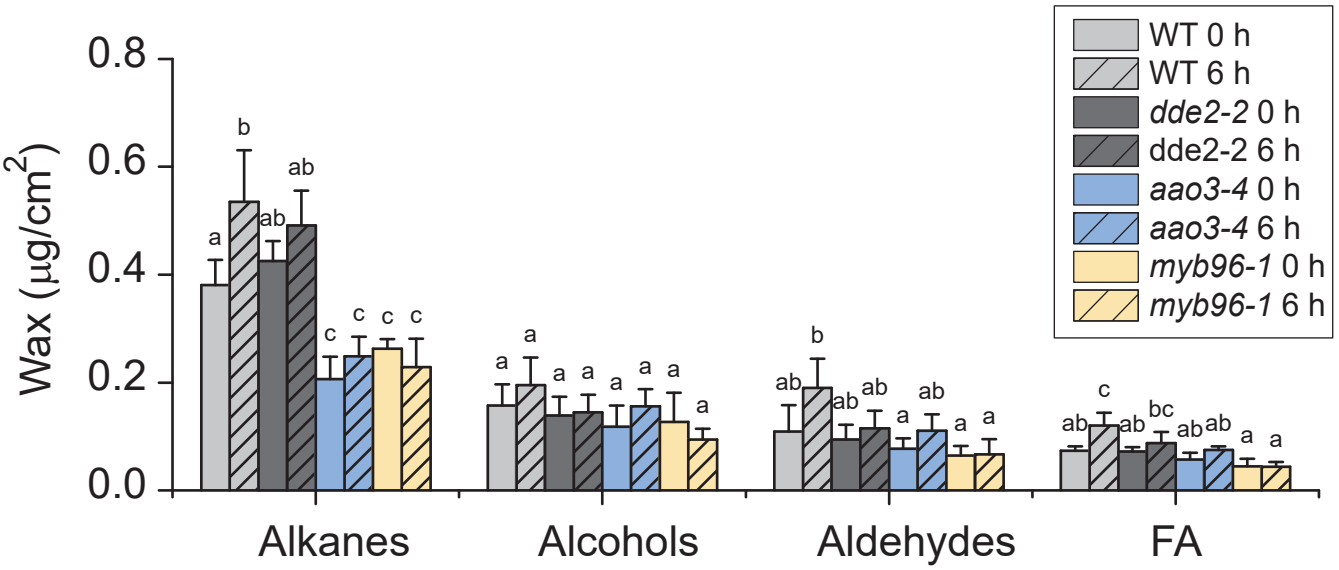

Figure S2. Wax alkanes, aldehydes, and fatty acids accumulate after wounding. Wax compound amounts before and 6 h after wounding. Each compound group was statistically analyzed separately. Letters indicate statistical significance determined by ANOVA and Tukey's post-hoc test (P<0,05). Values are means (±SD) of GC-FID analyses of plants harvested in three independent wounding experiments.

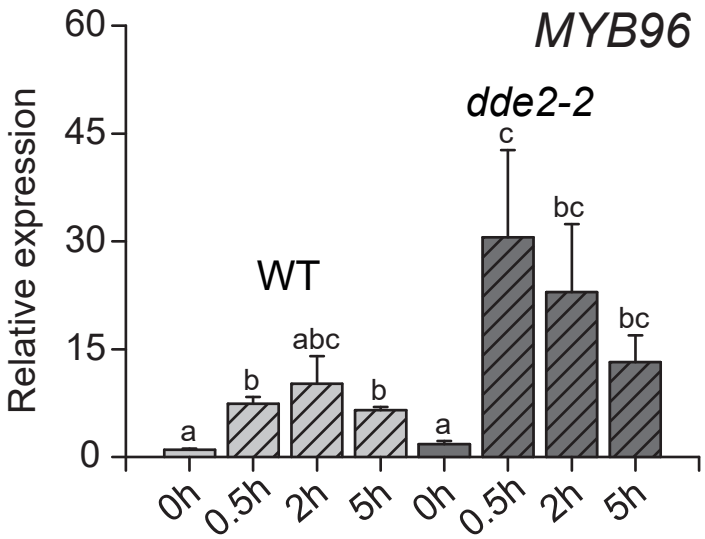

Figure S3. Expression of *MYB96* in WT and *dde2-2* before and after wounding. Letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values are means (±SE) of quantitative Real Time PCR analyses of plants harvested in three independent wounding experiments

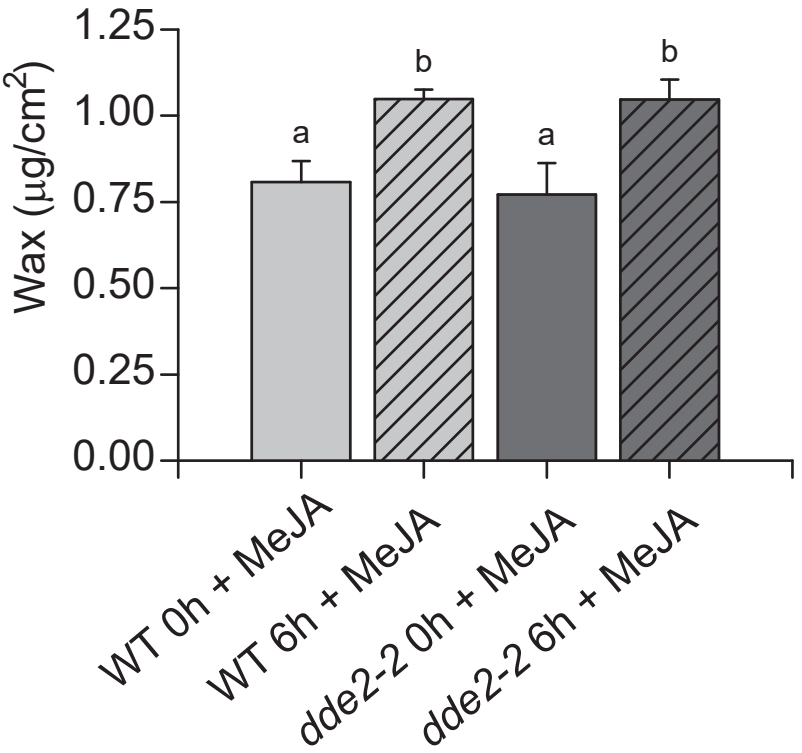

Figure S4. Wax analysis of plants sprayed with 5 mM MeJA before (0 h) and after wounding (6 h) in WT and the JA-deficient mutant *dde2-2*. Letters indicate statistical significance determined by ANOVA and Tukey's post-hoc test (P<0,05). Values represent means (±SD) of GC-FID analyses of plants harvested in three independent wounding experiments

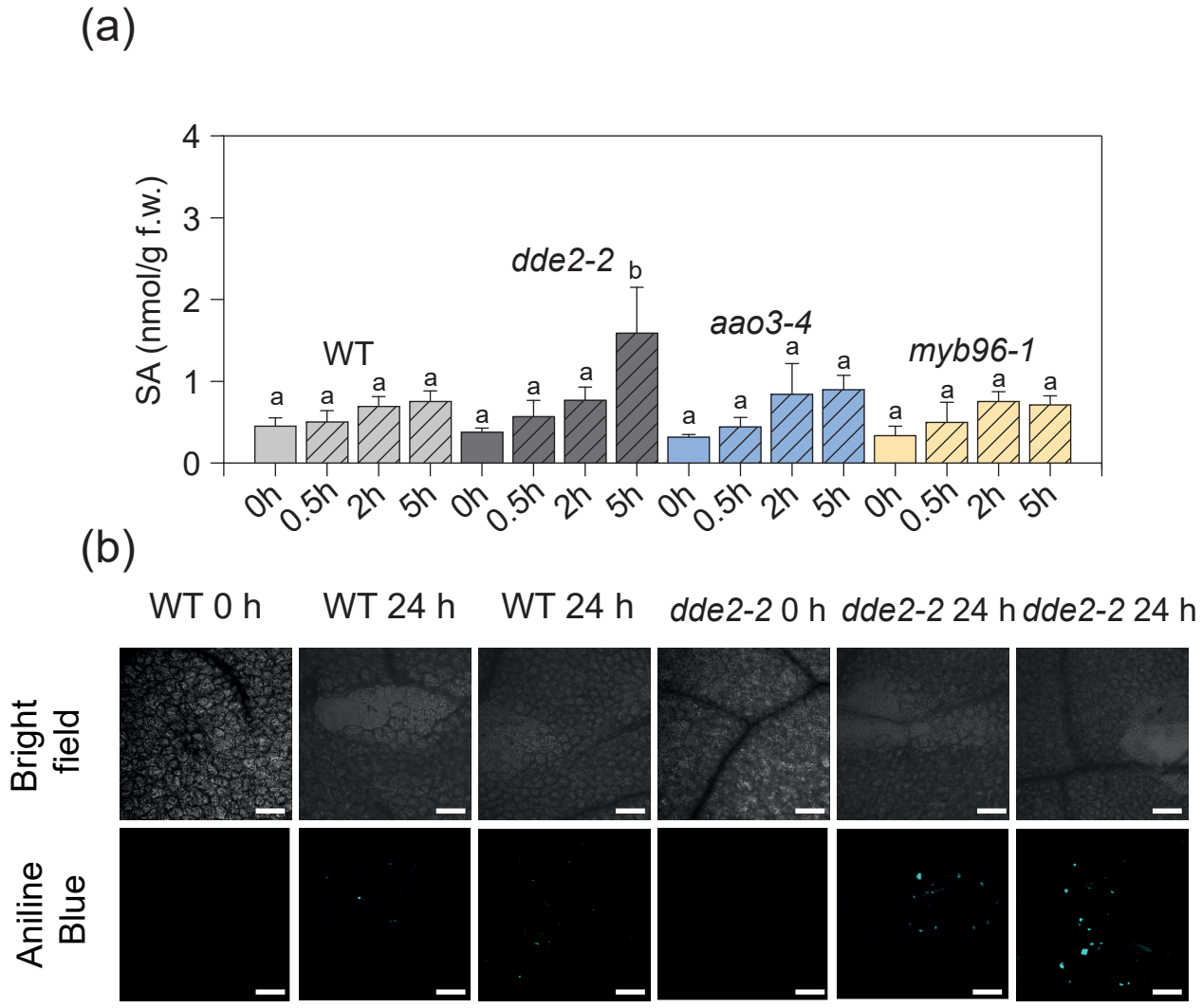

Figure S5. Analysis of SA content and callose deposition. (a) SA content in WT, d*de2-2, aao3-4* and *myb96-1.* Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of LC-MS/MS analysis of plants harvested at three independent wounding experiments. (b) analysis of callose formation in WT and *dde2-2* before and 24h after wounding. Analysis was performed by confocal microscopy, bars: 300 µm.

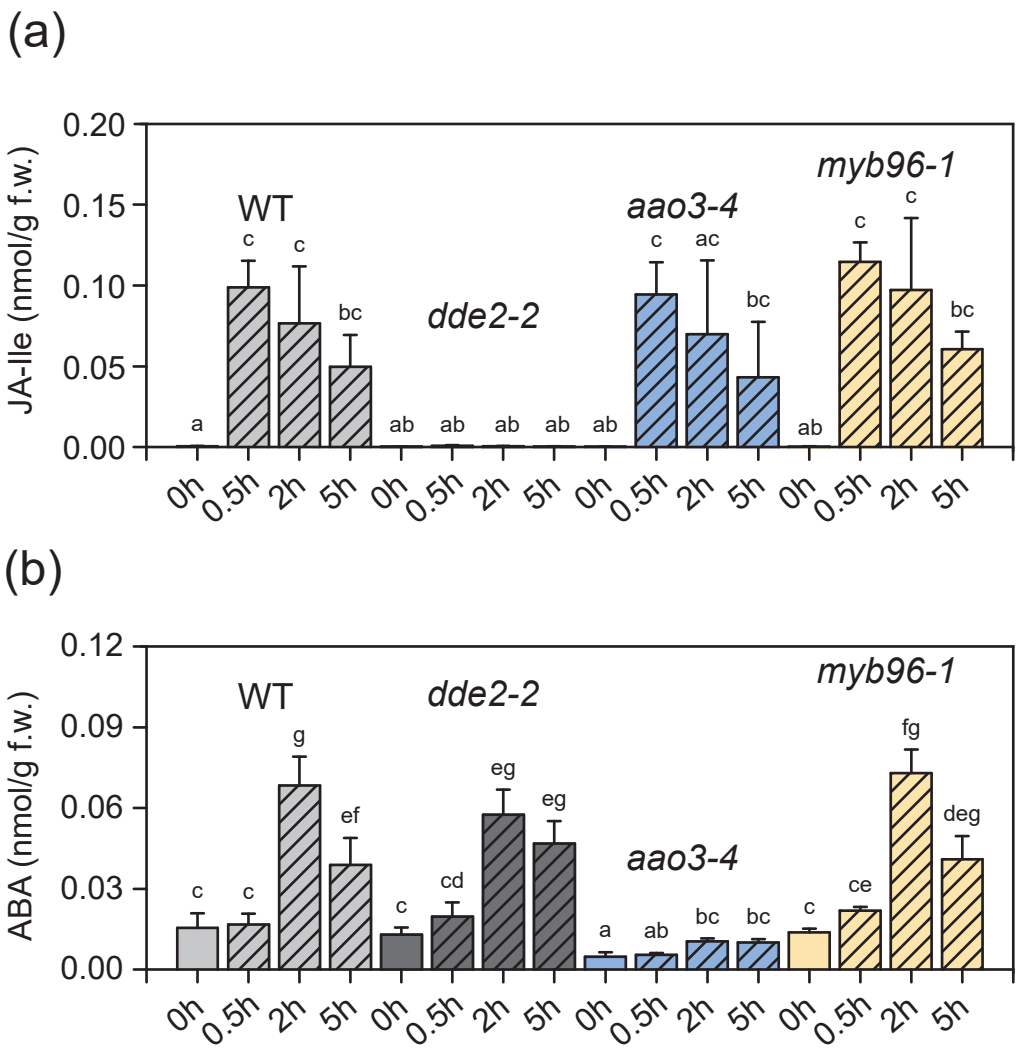

Figure S6. Analysis of JA-Ile and ABA amount in non-wounded and wounded leaves of WT, *dde2-2, aao3-4, myb96-1* before (0 h) and 0.5, 2, 5 hours after wounding. (a), JA-Ile amount and (b), ABA amount before and after wounding. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of LC-MS/MS analysis of plants harvested at three independent wounding experiments.

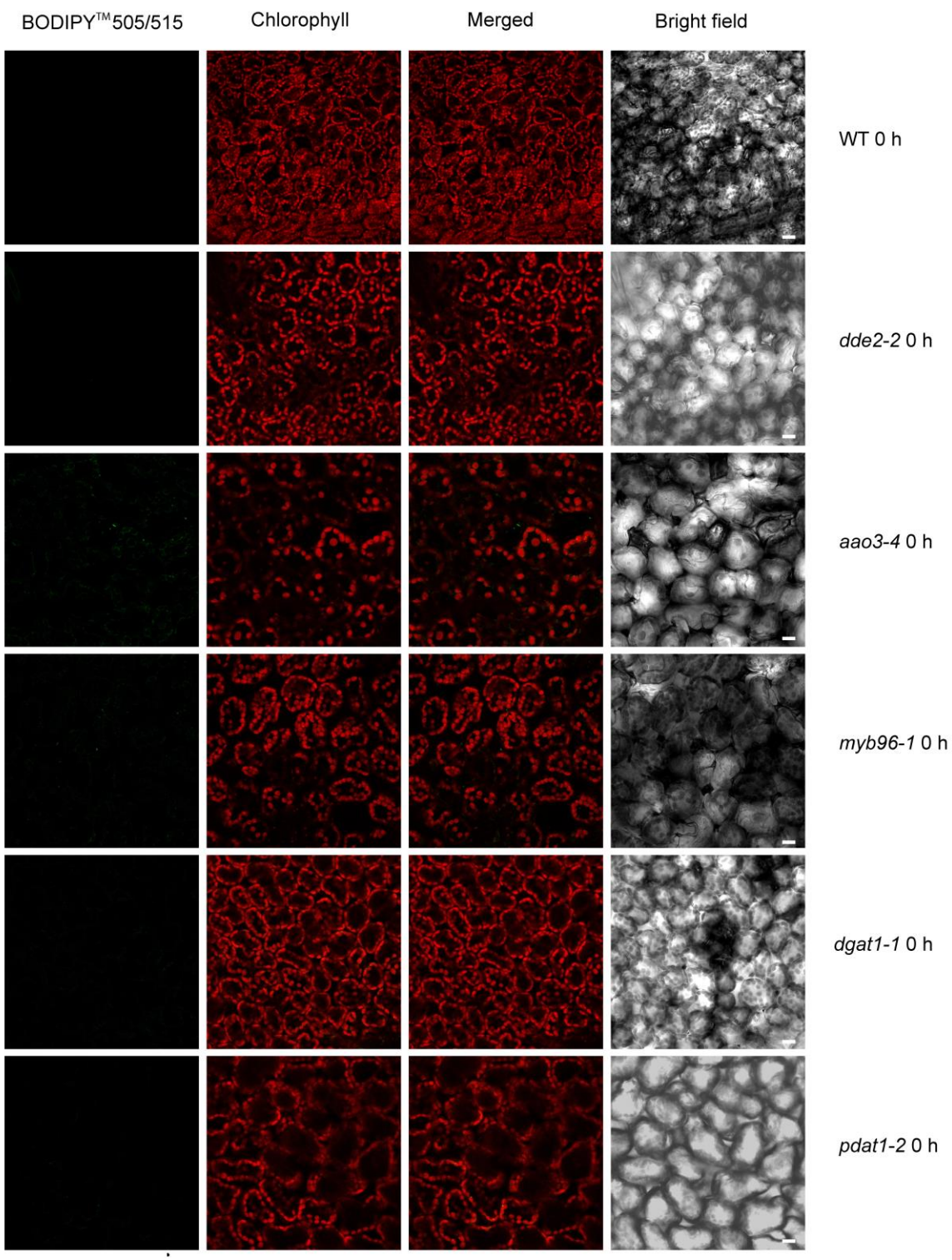

Figure S7. Lipid droplets formation analyzed by BODIPY staining (green) before wounding in WT, *dde2-2, aao3-4, myb96-1*, *dgat1-1* and *pdat1-2.* Analysis was performed by confocal microscopy, bars: 50 µm.

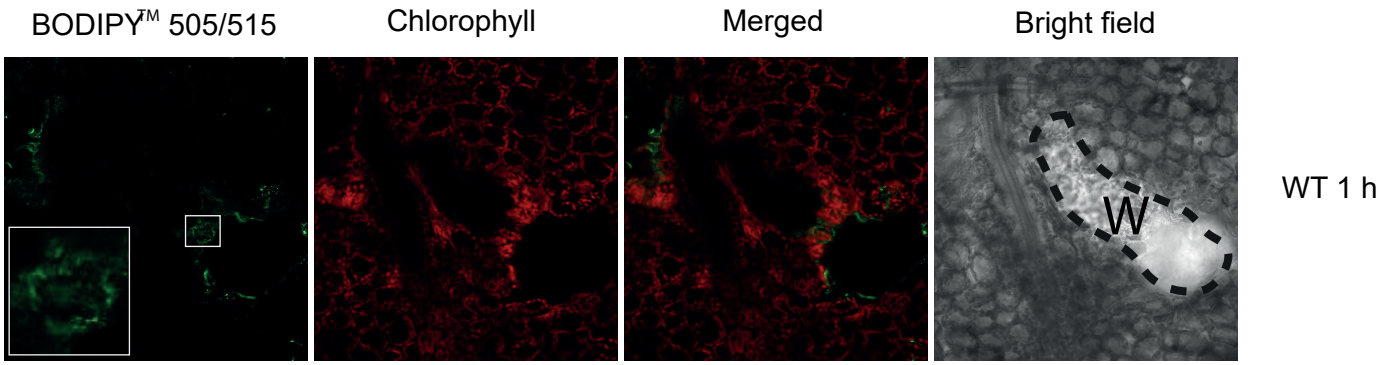

Figure S8. Lipid droplets formation analyzed by BODIPY staining (green) 1 hpw in WT. Analysis was performed by confocal microscopy.

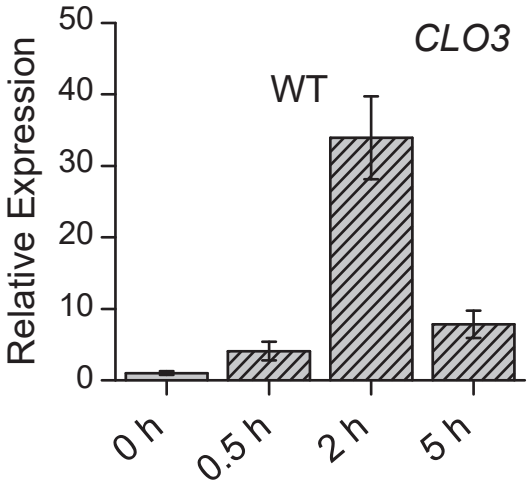

Figure S9. Expression of *CLO3* involved in lipid droplet formation in leaves. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of GC-FID analyses of plants harvested at three independent wounding experiments.

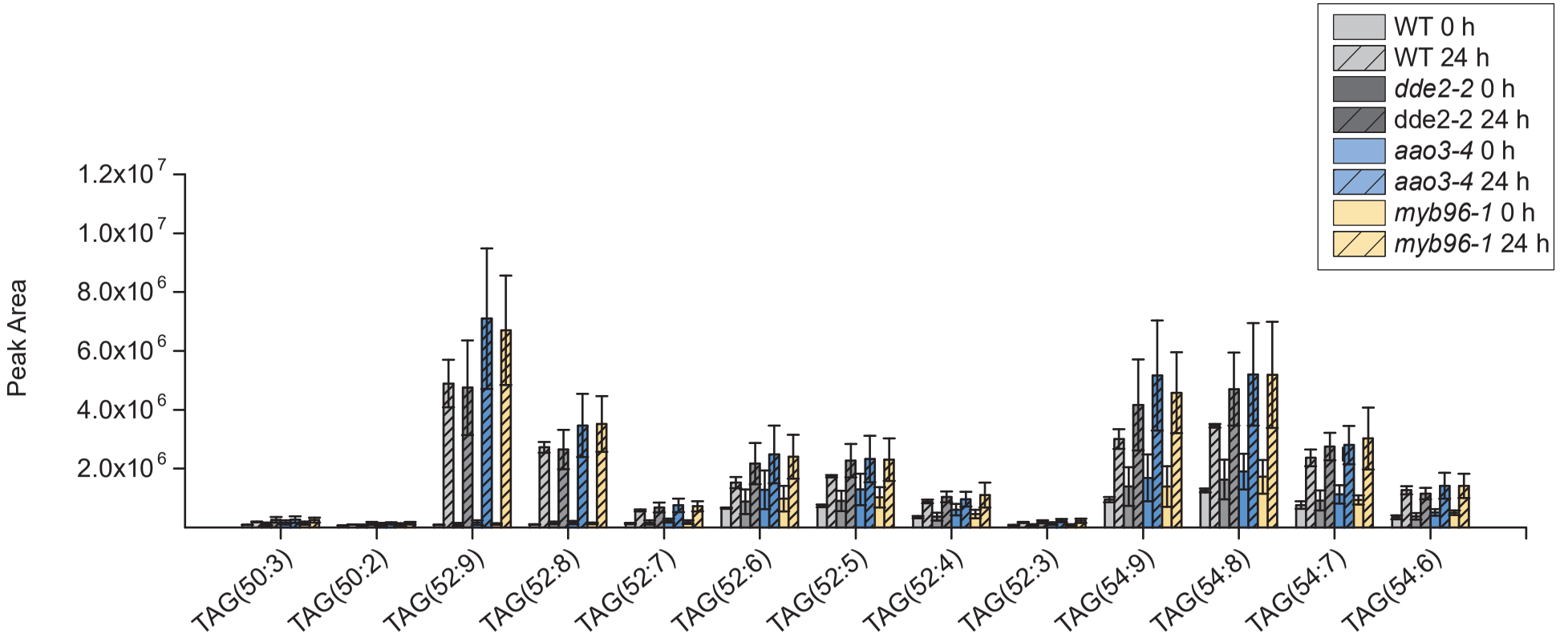

Figure S10. Triacylglycerol (TAG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

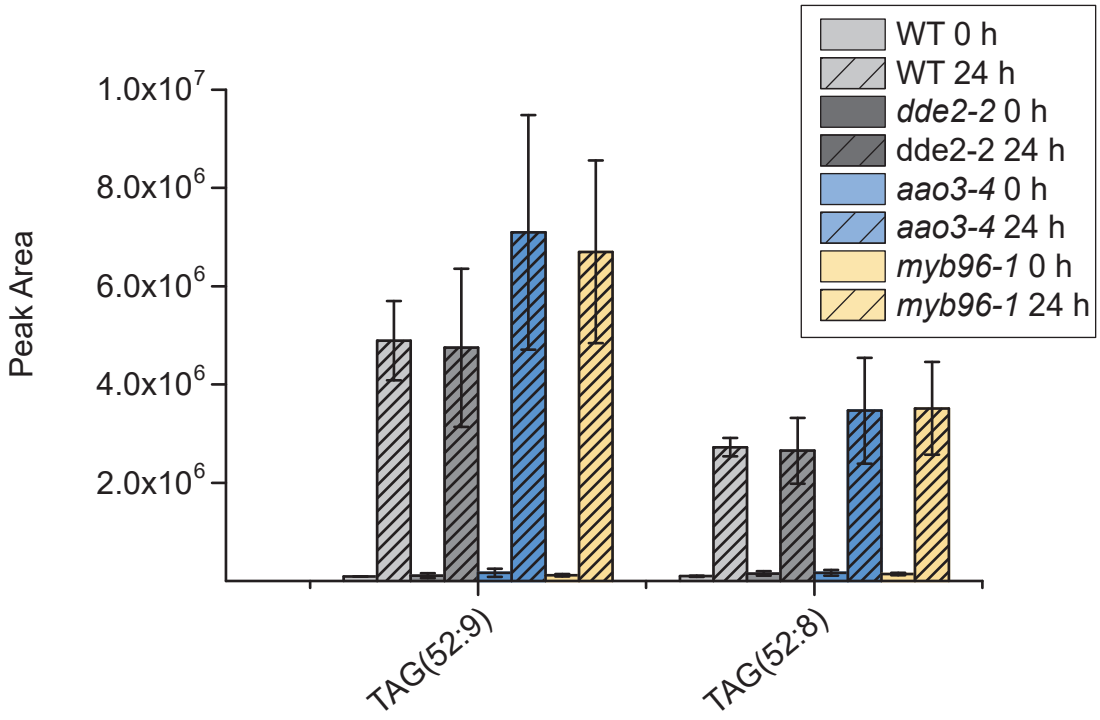

Figure S11. Chosen triacylglycerol (TAG) species before and 24 h after wounding in WT, *dde2-2, aao3-4, myb96-1*. Peak area of species 52:9 is a sum of areas of 16:3 and 18:3 while 52:8 is a sum of areas of 18:2,18:3,16:2 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

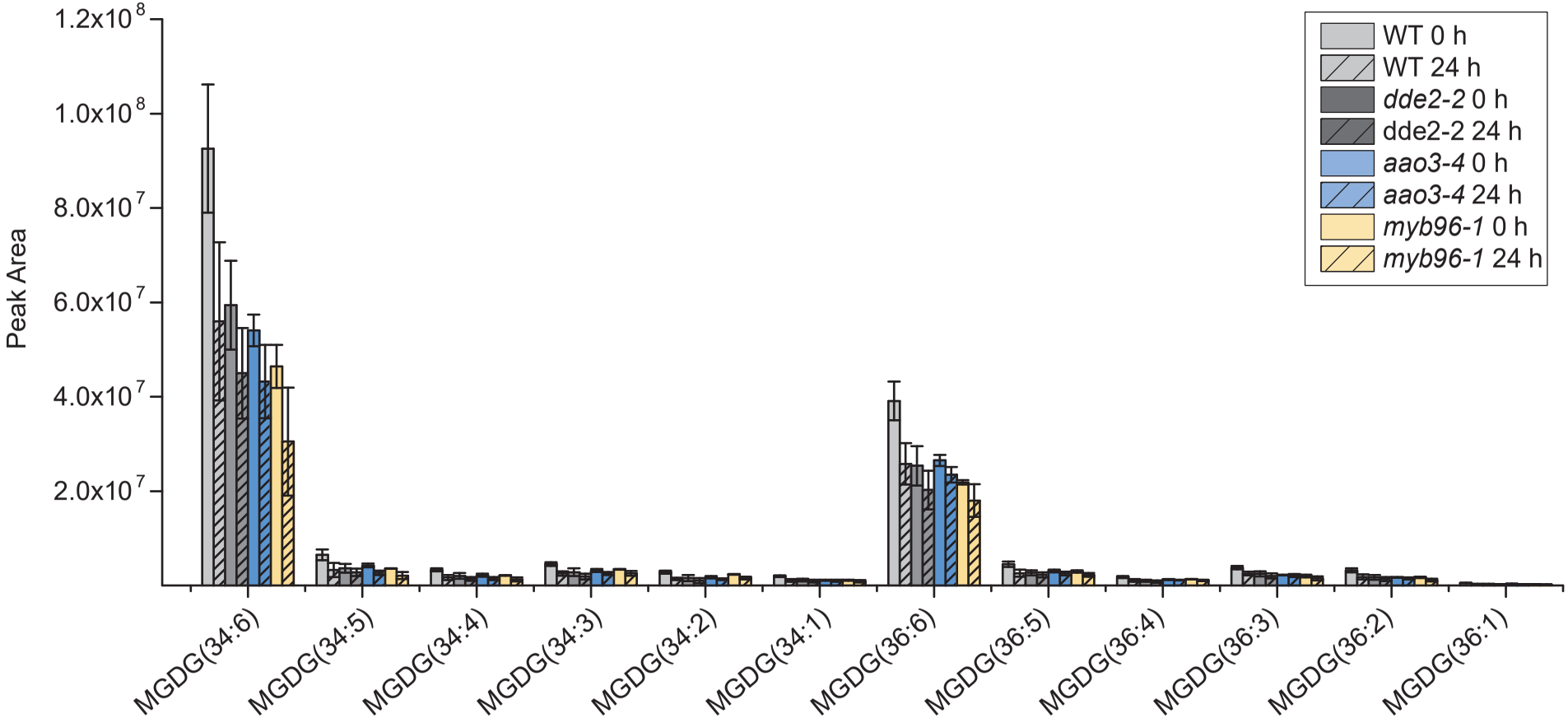

Figure S12. Monogalactosyldiacylglycerol (MGDG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

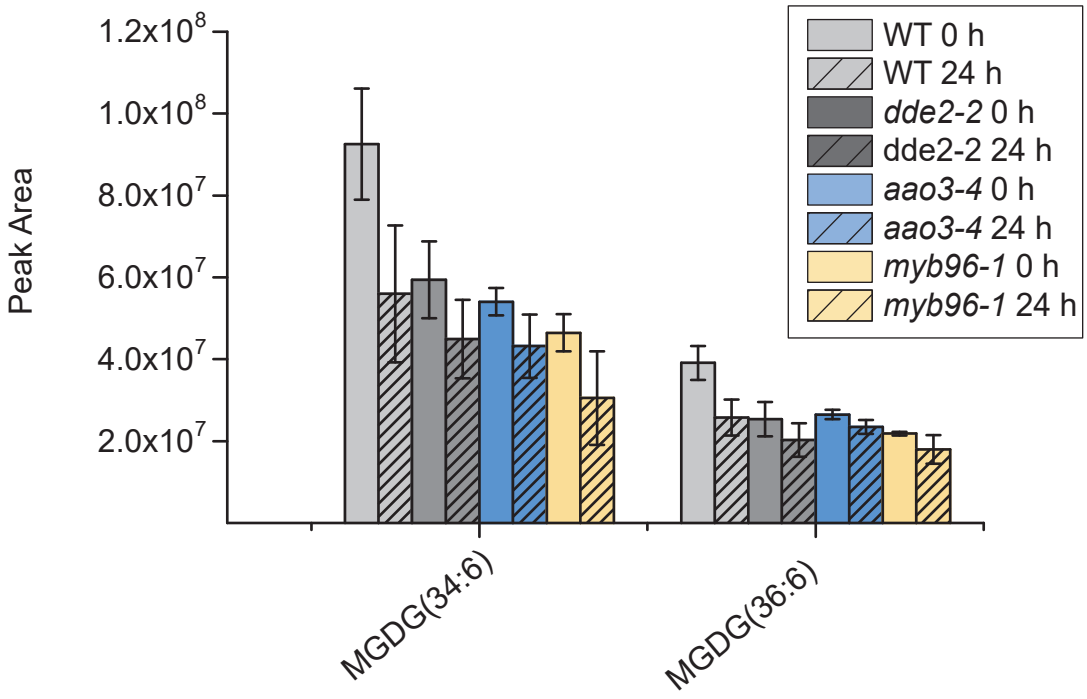

Figure S13. The most abundant monogalactosyldiacylglycerol (MGDG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Peak area of species 34:6 is a sum of areas of 16:3 and 18:3 while 36:6 is a sum of areas of 18:3 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

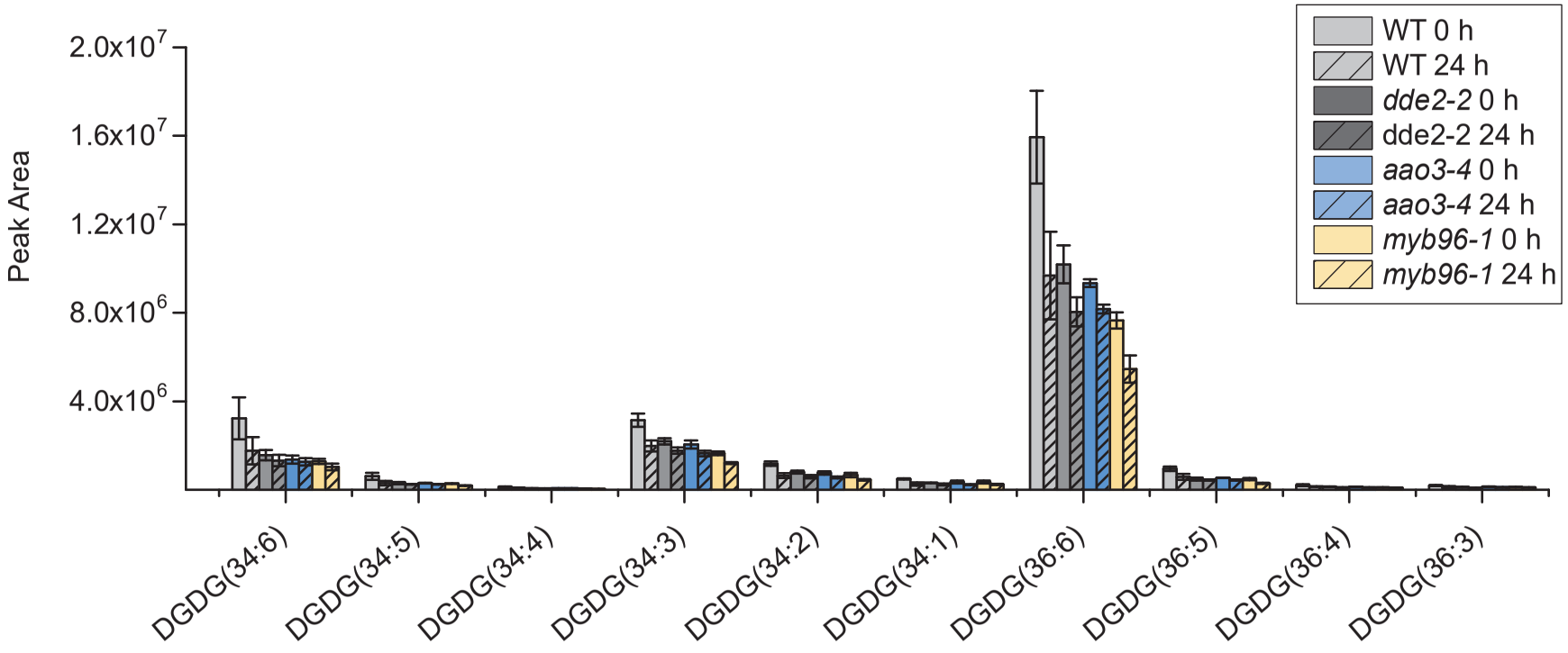

Figure S14. Digalactosyldiacylglycerol (DGDG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1.* Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

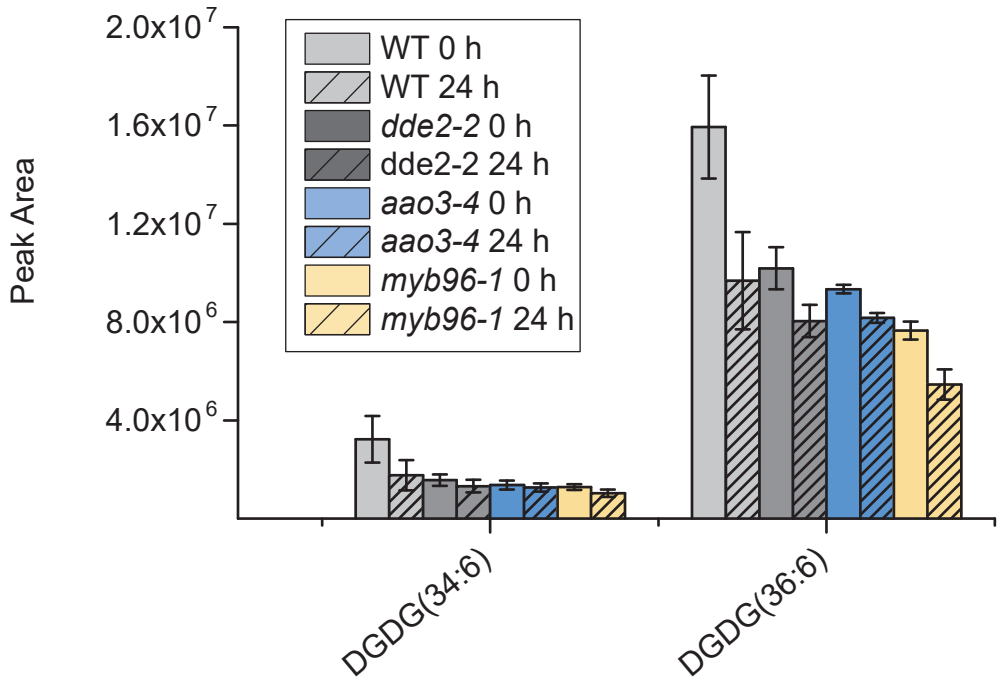

Figure S15. The most abundant digalactosyldiacylglycerol (DGDG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1.* Peak area of species 34:6 is a sum of areas of 16:3 and 18:3 while 36:6 is a sum of areas of 18:3 and 16:3. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

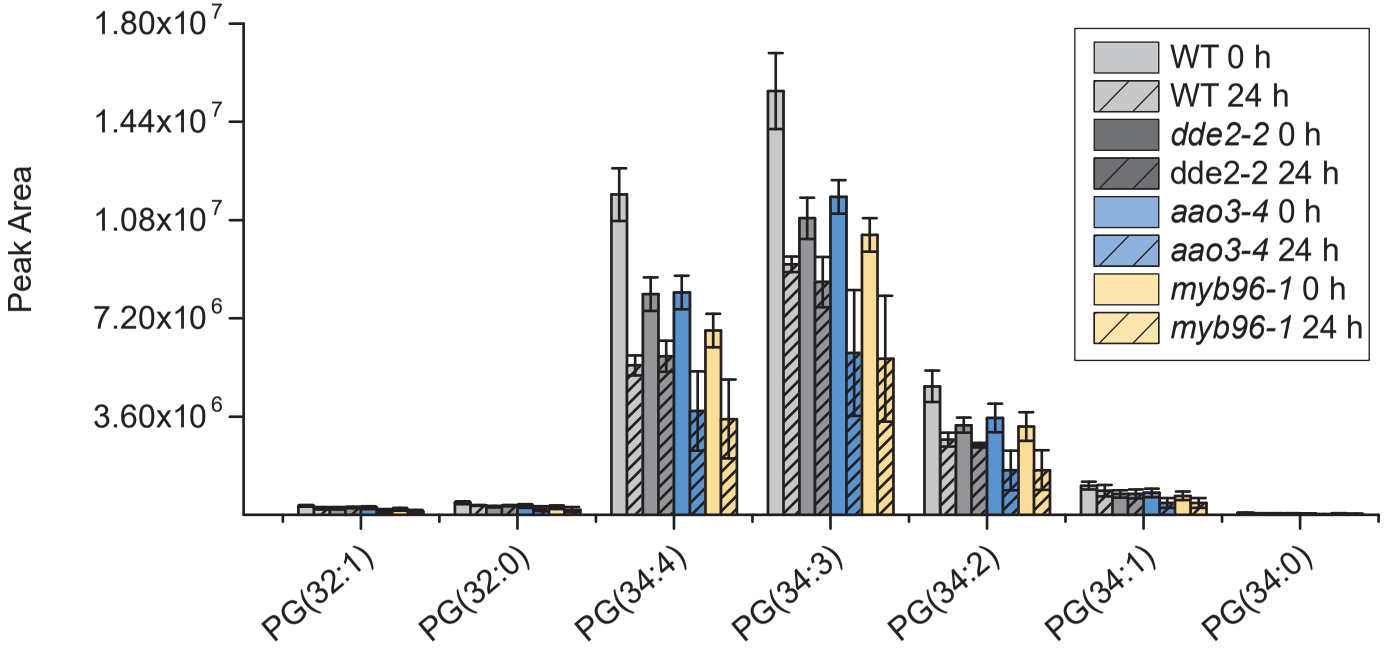

Figure S15. Phosphatidylglycerol (PG) composition before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

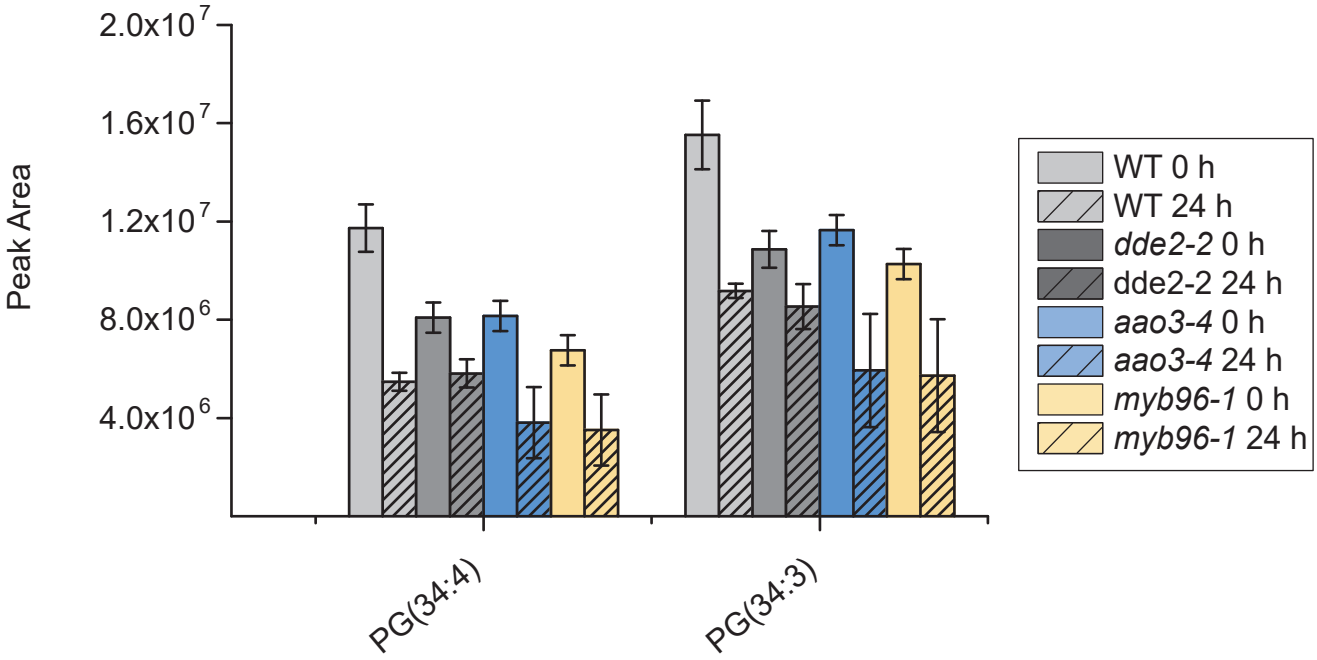

Figure S17. The most abundant phosphatidylglycerol (PG) species before and 24 h after wounding in WT, *dde2-2, aao3-4* and *myb96-1*. Peak area of species 34:4 is a sum of areas of 16:1 and 18:3 while 34:3 is a sum of areas of 16:1, 16:0, 18:3 and 18:2. Values represent means (±SD) of LC-MS/MS analyses of plants harvested at three independent wounding experiments.

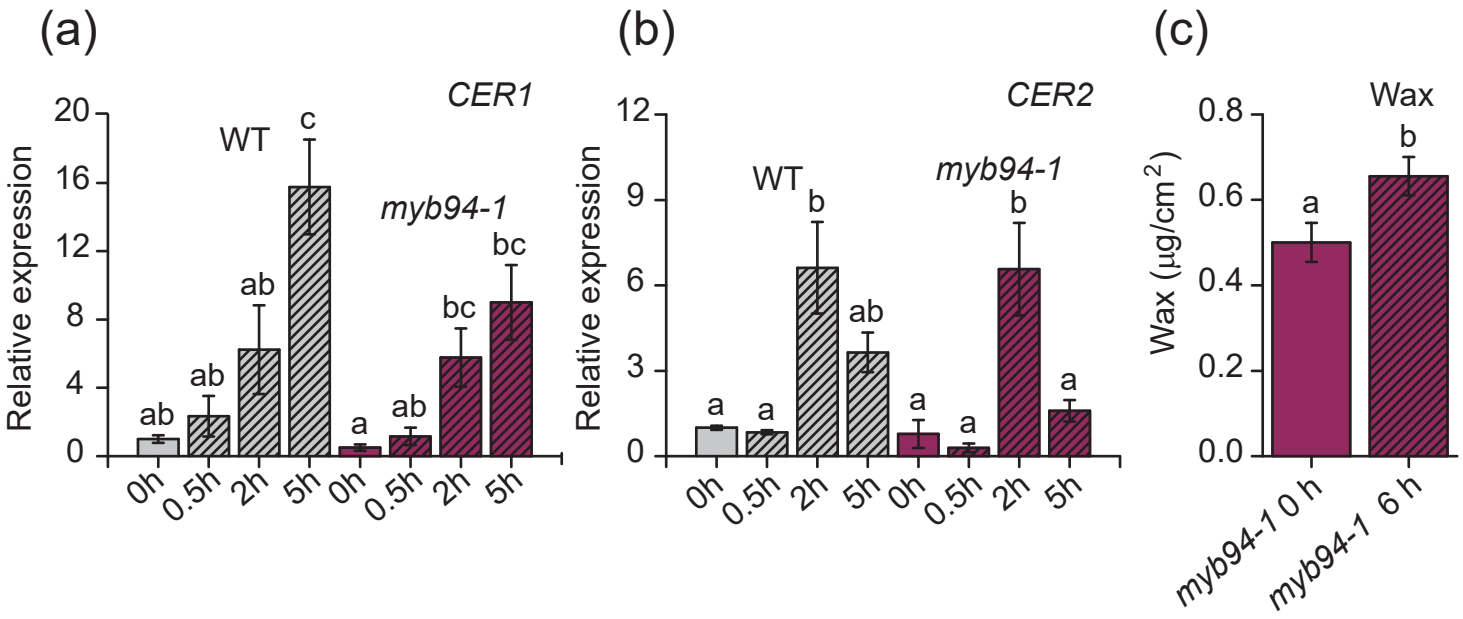

Figure S18. Response to wounding in *myb94-1*. (a) Expression of *CER1* and (b) *CER2* in WT and myb94-1 before (0 h) and 0.5, 2, 5 hours after wounding. Values represent means (±SE) of quantitative Real Time PCR analyses of plants harvested at three independent wounding experiments. (c), wax content before and 6h after wounding in *myb94-1*. Values represent means (±SD) of GC-FID analyses of 8 biological replicates

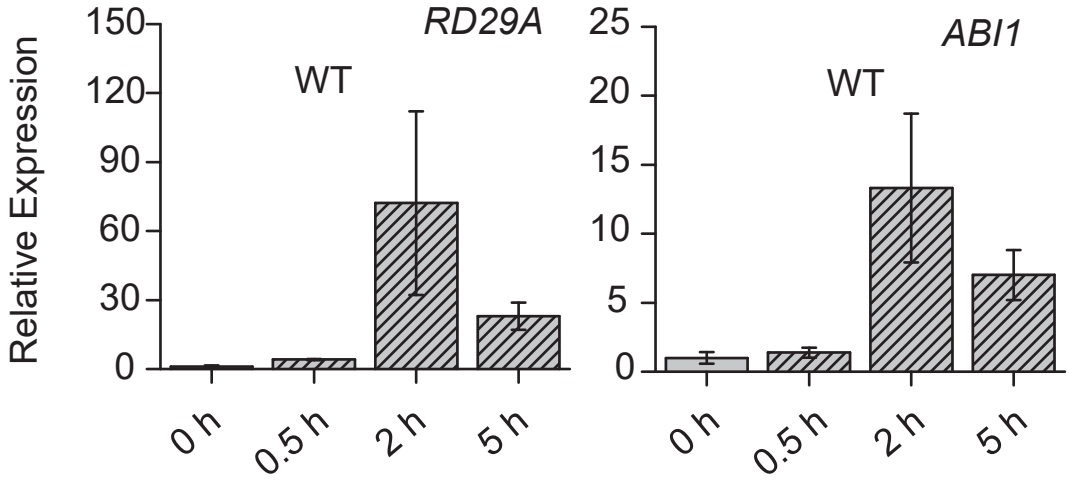

Figure S19. Expression of *RD29A* and *ABI1* involved in ABA signaling in abiotic stress response. Alphabetical letters indicate statistical significance determined by ANOVA and Tukey‘s post-hoc test (P<0,05). Values represent means (±SD) of qRT-PCR analyses of plants harvested at three independent wounding experiments

Gene name	Accession	Forward primer	Reverse primer
ACTIN8	AT1G49240	GGTTTTCCCCAGTGTTGTTG	CTCCATGTCATCCCAGTTGC
CER1	AT1G02205	GTATCTATCATACCACACCAGC	CCCATACTTGGTCCAAATCC
CER2	AT4G24510	GTCTACGATCACGTTCTTGG	CATCATTACGAGCATGAGAGG
CER3	AT5G57800	GGTTAACACCAAGAGAGCAG	CCAACCTCATGATGCTTCC
CER6	AT1G68530	GTGTTCGAAGGCCAGCTT	GAAGGACACGTTGGCATC
CER10	AT3G55360	CGAAGAAGAAGAGTGTGCG	CGACTGTTGCTGATCTGC
WSD1	AT5G37300	GATCAATGCTCCCAGATTCTC	CCCAAAGAGGTTTGGACATG
ABCG11	AT1G17840	CCGGGACAATCTGTTACTTC	GCCTGAAGAATCCAGAAACC
LTPG2	AT3G43720	GCTAAAGCCGTTGGTCCC	GAAACATCCGCTAGTAGCTTC
DGAT1	AT2G19450	CCACGTTCTGCATGTATACG	GTAGAACATGCAGAGCCAC
PDAT1	AT5G13640	GACAAGACAAACAAACATGGC	CCTGTCAGCTTATGTGAATCC
MYB96	AT5G62470	CACGCTTAATACCGGGTC	GACACCAGAGGAAGACATC
ABI1	AT4G26080	CCGCAGGAGGGAAAGTGATT	TGCCATCTCACACGCTTCTT
RD29A	AT5G52310	CACAATCACTTGGCTCCACTGTTG	ACCTAGTAGCTGGTATGGAGGAACT
CLO3	AT1G70670	TACACTTCCGAGTTGGGTGC	ACTGGGACATACCTTCCTTCG

Supplemental Table 1. Primers used in the study.

Chapter 3: MYB41 is a negative regulator of wax biosynthesis in inflorescence stems of *A.thaliana.*

The article is prepared for submission. The supplemental materials are attached at the end of the chapter.

Author contribution

Milena Lewandowska designed all experiments. She created all transgenic lines used in this study, performed phenotype analysis of all overexpression lines and organ specific expression pattern of *MYB41* via qRT-PCR. The phenotypic analysis of the *myb41* mutant collection she performed either with or was performed by a master student guided by her instructions. She analyzed the data, displayed and interpreted the results and wrote the first draft of the manuscript.

MYB41 is a negative regulator of wax biosynthesis in inflorescence stems of *A. thaliana*.

Milena Lewandowska[1], Alisa Keyl[1], Ljerka Kunst[3] and Ivo Feussner[1,2,4]

[1]*Univeristy of Goettingen, Albrecht-von-Haller-Institute for Plant Sciences, Department of Plant Biochemistry, Goettingen, Germany.*

[2]*Univeristy of Goettingen, Goettingen Center for Molecular Biosciences (GZMB), Service Unit for Metabolomics and Lipidomics, Goettingen, Germany.*

[3]*University of British Columbia, Department of Botany, Vancouver, Canada.*

[4]*Univeristy of Goettingen, Goettingen Center for Molecular Biosciences (GZMB), Department of Plant Biochemistry, Goettingen, Germany.*

ABSTRACT

Aerial parts of plants are covered by the cuticle, a hydrophobic layer composed of cutin and wax, which prevents loss of water and pathogen attack. The belowground parts of plants are covered with suberin, which has similar properties as the cuticle. The regulation of cutin, wax and suberin biosynthesis is extensively studied and transcription factors regulating those pathways are characterized. *MYB41* (At4g28110) has been described recently as a putative positive regulator of wax and suberin biosynthesis. MYB41 belongs to the R2R3-MYB transcription factor family, which is defined by two MYB repeats named R2 and R3. Knockout mutants of *MYB41* were not described so far. In this study, we characterized MYB41 and its biological role in Arabidopsis inflorescence stems, using a mutant collection obtained via CRISPR/Cas9 approach. Our analysis revealed that MYB41 is a negative regulator of wax biosynthesis in inflorescence stem. More specific, it reduces the amount of primary alcohols by suppressing the expression of the main fatty acid reductase in inflorescence stems, *CER4/FAR3*. Moreover, we show that both, the C-terminal part of the protein as well as the R2 domain were essential for the proper function of MYB41 and only the R2 domain was sufficient to repress primary alcohol biosynthesis. Our study reports a specific function of the MYB41 transcription factor on the regulation of wax biosynthesis.

INTRODUCTION

The first land plants developed hydrophobic barriers to adapt to the non-aqueous environment. The cuticle is covering all aerial parts of plants. It consists of cutin and waxes. Cutin is a fatty acid and glycerol based polyester forming the scaffold of the cuticle (Fich et al., 2016). Wax is a mixture of aliphatic compounds surrounding and covering cutin and therefore the surface of above-ground part of plants (Samuels et al., 2008). In contrast to that are the belowground part of plants, seed coat and tree bark covered by suberin, a lipidic barrier consisting of an aliphatic and phenolic polymer (Franke and Schreiber, 2007).

The composition of cuticular wax differs in various organs. In Arabidopsis leaves alkanes, primary alcohols, aldehydes and very long chain fatty acids (VLCFA) are present in its structure, however in inflorescence stems and siliques the major components are ketones and secondary alcohols, barely detectable in leaves (Suh et al., 2005; Bernard and Joubès, 2013). Wax is synthesized in ER of epidermal cells and this process starts with the elongation of C16 or C18 acyl-CoAs (Suh et al., 2005). The fatty acid elongase (FAE) complex consist of ß-keto acyl-CoA synthase, ß-keto acyl reductase, hydroxyacyl-CoA dehydratase and enoyl-CoA reductase. VLCFA obtained in this step can be channeled into two pathways: the alcohol or alkane forming pathway (Samuels et al., 2008). In the alcohol-forming pathway, VLCFA are reduced to primary alcohols by specific fatty acyl-CoA reductases (CER4/FAR3 or FAR6) or condensed to form wax esters by WAX SYNTHASE/ACYL-COA:DIACYLGLYCEROL ACYLTRANSFERASE1 (WSD1) (Suh et al., 2005; Rowland et al., 2006; Li et al., 2008). Whereas in the alkane-forming pathway, a CER1/CER3/CYTB5 complex is converting VLCFA to alkanes and aldehydes which can be further oxidized to ketones and secondary alcohols by MIDCHAIN ALKANE HYDROXYLASE1 (MAH1) (Greer et al., 2007; Bernard et al., 2012). All wax components are exported across the plasma membrane and the cell wall by the ATP-binding cassette transporter (ABCG11/CER5 and ABCG12) or glycosylphosphatidylinositol-anchored lipid transfer proteins (LTPG1 and LTPG2) (Bird, 2008; DeBono et al., 2009; Kim et al., 2012).

Environmental factors, hormones and transcription factors influence wax biosynthesis and deposition. Wax biosynthesis is induced by drought and abscisic acid (Kosma et al., 2009; Seo et al., 2009) and is diminished in dark and high humidity (Baker, 1974; Go et al., 2014; Kim et al., 2018). Different transcription factors are involved in the

regulation of those processes. So far, two negative regulators were described – DECREASE WAX BIOSYNTHESIS1 and 2 (DEWAX1 and DEWAX2) (Go et al., 2014; Kim et al., 2018). Both transcription factors down-regulate wax biosynthesis in the dark. The first described positive regulator is SHINE1/WAX INDUCER1 (SHN1/WIN1). It controls genes early in the pathway from the FAE as well as the alkane-forming pathway (Aharoni et al., 2004). Other positive regulators of wax biosynthesis are belonging to ABA dependent R2R3-MYB transcription factors. MYB94 is inducing the expression of genes from the FAE as well as the alcohol-forming pathway – *CER4/FAR3* and *WSD1* (Lee and Suh, 2014). MYB96, another ABA dependent R2R3-MYB transcription factor, induces wax biosynthesis during drought and pathogen attack. Overexpression of MYB96 led to higher drought resistance (Seo et al., 2009; Seo et al., 2011; Lee et al., 2016). A third ABA inducible transcription factor is MYB41 (Cominelli et al., 2008). Its transcription was found to be induced by salt, drought, ABA and cold treatment. (Cominelli et al., 2008). MYB41 is down-regulating genes involved in salinity stress response, suggesting that it may act as a transcriptional respressor (Lippold et al., 2009). In plants overexpressing MYB41, repression of other MYB trancription factors was observed under high salinity, e.g. MYB9 involved in suberin biosynthesis or MYB52 negatively regulating pectin biosynthesis in the seed coat (Lippold et al., 2009; Lashbrooke et al., 2016; Shi et al., 2018). Moreover, overexpression of MYB41 led to the appearance of suberin-like lamellar structures and higher wax content, especially alkanes, VLCFA and primary alcohols in Arabidopsis leaves. However, primary alcohols were enriched only in a suberin-like type (with less than 24 carbons), where the amount of cuticular wax-type alcohols were reduced or unchanged (Kosma et al., 2014). There are two current models for the mode-of-action of MYB41, which are contradictory to each other. One is suggesting that MYB41 is a negative regulator of short-term salinity response and the second one proposes that MYB41 might be a positive regulator of wax and suberin biosynthesis (Kosma et al., 2014; Vishwanath et al., 2015)

So far, the research about MYB41 was focused on its gain-of-function lines, since there were no loss-of-function lines available (Cominelli et al., 2008; Lippold et al., 2009; Kosma et al., 2014). In this study, we further analyzed the biological role of the MYB41 transcription factor by employing knockout lines generated by a CRISPR-Cas9 approach. Moreover, with this mutant collection we were able to identify domains within the sequence of MYB41 being necessary for its function. Its C-terminus as well as the

R2 DNA binding domain play an important role on repressing the expression of *CER4/FAR3*, encoding for the main fatty acyl-CoA reductase, and in consequence of primary alcohol biosynthesis in Arabidopsis inflorescence stems.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

Following lines were used in described experiments: *Arabidopsis thaliana* Columbia-0, mutants of MYB41 – *myb41-1, myb41-2, myb41-3* and *myb41-4* obtained by CRISPR-Cas9 using pHEE401E plasmid with egg specific promotor as described (Wang et al., 2015). sgRNA sequences are shown in Supplemental Table 1. MYB41-OX and MYB41-R2-OX lines were cloned with using primers shown in Supplemental Table 1. The PCR product was cloned in pUC18-Entry between 35S Cauliflower Mosaic virus promoter and 35S polyA terminator and afterwards cloned into Gateway compatible pCAMBIA3300.Gc vector suitable for glufosinate ammonium selection (Hornung et al., 2005) and transformed to *myb41-2* or WT (Col-0). All plants were grown on soil under following conditions, unless specified otherwise: white light illumination (130-150 µmol m^{-2} s^{-1}) under long day conditions (16 h light : 8 h dark) with at 22 °C during day and 18 °C during night with approximately 60 % humidity.

Stress assays

Plants were grown as described above and 5 or 6 days prior to harvesting were transferred to black plastic box (dark stress) or to chamber with approx. 80 % humidity at night and 85 % humidity at day, respectively. For ABA treatment, 8-days old seedlings were transfered to petri dishes with 10 µM ABA (Sigma-Aldrich) diluted in water:methanol (90:10, v:v) or mock water:methanol (90:10, v:v). After 24 h of incubation, seedling were frozen in liquid N_2.

Expression analysis

Total plant RNA was extracted with SPECTRUM™ Plant Total RNA Kit (Sigma-Aldrich). RNA quantification was measured via NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). RNA was treated with DNase I (Thermo Fisher Scientific) and cDNA was synthesized using Revert Aid H Minus Reverse Transcriptase (Thermo Fisher Scientific). qRT-PCR was performed using Takyon No ROX SYBR Mastermix

blue dTTP (Kaneka Eurogentec) in a reaction volume of 20 µl. The gene *ELONGATION FACTOR1* (AT1G30230) was used as a control. Analysis was performed in iQ5 real time detection system (Bio-Rad). All primers used in this experiment are listed in Supplemental Table 1.

Wax analysis

Wax was extracted from leaves and inflorescence stems of 4-week old plants, while wax from siliques was extracted from 3-4 siliques (each 1.5 cm long) of 5-week-old plants. Inflorescence stems, leaves or siliques were immersed in chloroform containing tetracosane (Sigma-Aldrich) as an internal standard. Samples were dried under N_2 stream and redissolved in 10 µl of N,O-bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich) and 10 µl of pyridine (Sigma-Aldrich). Derivatization was performed in 80 °C for 1 h. Afterwards samples were dried under N_2 stream and redissolved in 10 µl of chloroform. These were quantified by gas chromatography with flame ionization detector (Agilent GC 6890, Agilent Technologies) coupled with a 30-m HP-1 column using helium as a gas carrier. 2 µl of each sample was injected with split 1:5 for leaves and siliques samples and 1:15 for inflorescence stems samples. Gas chromatography was carried out with oven temperature set at 50 °C for 2 min then raised by 40 °C min^{-1} to 200 °C and then held for 1 min, afterwards raised by 3 °C min^{-1} up to 320 °C and then held for 15 min. The signals were integrated using the ChemStation Software (Agilent Technologies). Wax compounds were identified beforehand with gas chromatography linked with mass spectrometric detector (Agilent 5973 Network, Agilent Technologies). Quantification of the wax amount was determined by comparing the peak areas from ionization detector to the internal standard. Leaves and inflorescence stems areas were determined as described (Haslam and Kunst, 2013).

Cutin monomer analysis

Leaves and inflorescence stems of 4-week-old plants were used in this experiment. 300 mg of homogenized leaves or inflorescence stems were added to 1-proponol at 60 °C and afterwards shaken for 4 h at 4 °C. Next, the 2-propanol was replaced by fresh 2-proponol and shaken overnight. The next day the pellet was washed with chloroform/methanol 2:1 (v/v) for 8 h at 4 °C and afterwards with chloroform/methanol 1:2 (v/v) at 4 °C overnight. Afterwards the pellet was dried under N_2 stream. The dried

residue was extracted with fallowing solvents: methanol for 30 min, H_2O for 30 min, 2 M NaCl for 1 h, H_2O for 30 min, methanol for 30 min, chloroform/methanol 2:1 (v/v) overnight, chloroform/methanol 1:2 (v/v) overnight. Next, the pellet was dried under N_2 stream and lyophilized for remaining water removal. The dried and lyophilized pellet was methanolized (6 mg for leaves and 4 mg for roots) with 1 M methanolic HCl (Sigma-Aldrich) for 2 h at 80 °C. Prior to methanolysis fallowing standards were added 6 µg each: 17:0 fatty acid, 15:0 ω-OH fatty acid (all Sigma-Aldrich). After methanolysis the samples were extracted 3 times with hexane. The collected hexane was combined and dried. The leaves and inflorescence stems residue was dissolved in 4 µl hexane with 2 µl N,O-bis(trimethylsilyl)trifluoroacetamide. GC-FID analysis was done using a capillary HP-5 column (30 m x 0.25 mm, 0.25 µm coating thickness). Helium was used as carrier gas with a flow of 1 ml min^{-1}. The temperature gradient was as follows: 150 °C for 1 min, 150-200 °C at 4 °C min^{-1}, 200-330 °C at 5 °C min^{-1} and 330 °C hold for 6 min. 2 µl of each sample were injected with a split ratio of 5:1. Cutin monomers were identified beforehand by GC/MS and their amount was determined by comparing the peak areas from ionization detector to the internal standards.

RESULTS

Four mutants of *MYB41* were generated by CRISPR/Cas9

MYB41 is a protein, which belongs to the subgroup 11 of the R2R3-MYB transcription factor family. It contains two DNA binding domains, R2 and R3 (Fig. 1A). In addition, all members of the subfamily 11 harbor the same amino acid motif, located from position 127 to 134, which contains 7 conserved amino acids (Fig. 1A). It might be a putative activation or repression domain (Stracke et al., 2001; Dubos et al., 2010). It is known, that the serine at position 251 (Ser^{251}) of MYB41, which represents a phosphorylation site, is necessary for binding to the promotor of uncharacterized *LIPID TRANSFER PROTEIN* (At3g2262) (Hoang et al., 2012). Using a CRISPR/Cas9 approach under control of an egg cell-specific promotor, we were able to obtain four homozygous mutants of *MYB41* in the T_1 generation with an efficiency for the mutation of approx. 7 % of all transformants. Two single guide RNAs (sgRNAs) were designed, of which one targets the R2 DNA binding domain and the second one the C-terminal coding region of MYB41. Two of the obtained mutant alleles, *myb41-1* and *myb41-2,* have a mutation directly in the R2 DNA binding domain (Fig. 1B, Supplemental Fig. 1).

myb41-1 has a deletion of 6 nucleotides (Supplemental Fig. 2), which results in a lack of two amino acids in the R2 domain, however the rest of the protein remained unchanged. In *myb41-2*, an insertion of a single nucleotide resulted in a shift of the open reading frame leading to a pre-mature stop codon (Fig. 1B, Supplemental Fig. 1). For that reason, it encodes only a very short protein of 47 amino acids (Supplemental Fig. 2). The most intriguing mutation happened in *myb41-3* where presumably, both sgRNA targeted the gene sequence. As the result, the sequence between the two sgRNAs was cut out and re-inserted in reverse direction (Supplemental Fig. 1). This resulted in a frame shift and a premature stop codon. The protein in *myb41-3* consists of 41 amino acids (Supplemental Fig. 2). The fourth mutant, *myb41-4* shows a deletion of 27 nucleotides corresponding to a deletion of 9 amino acids and a protein of 271 amino acids (Supplemental Figs. 1, 2). The mutation led to a shift of Ser^{251} to Ser^{242} in *myb41-4* (Fig. 1A, Supplemental Fig. 2). The R2 and R3 domains remained unchanged in the *myb41-4* mutant.

MYB41 shows strong expression in roots

Previous research on MYB41 did not include an analysis of its expression pattern. Quantitative Real-Time PCR (qRT-PCR) revealed that *MYB41* is expressed in all plant organs analyzed. The highest expression was observed in roots, an organ rich in suberin (Fig. 2). *MYB41* is also expressed in all aerial parts of the plant (flowers, siliques, stem, cauline and rosette leaves), suggesting that it may be involved in the regulation of both cutin and wax biosynthesis. Interestingly, the *MYB41* expression pattern differed in the inflorescence stem between top and base stem. The expression in the top of the stem was lower than in the basal part, although extensive wax biosynthesis takes place in the top part. All *myb41-(1-4)* mutant alleles had no visible phenotype on any of mentioned organs.

MYB41 is a negative regulator of primary alcohol biosynthesis in Arabidopsis inflorescence stems

To investigate the role of MYB41 in wax rich organs like leaves, inflorescence stems and siliques we decided to asses the content of those compounds in MYB41 mutants. First, the wax load on inflorescence stems surface was examined via gas chromatography with flame ionization detector (GG-FID). The analysis revealed that

the total amount of surface wax is significantly higher in inflorescence stems of three out of the four *myb41* mutant alleles, namely *myb41-1*, *myb41-3* and *myb41-4* (Fig. 3A). It was a surprising finding, especially due to the fact that MYB41 was classified as a positive regulator of wax biosynthesis (Kosma et al., 2014). The analysis of the wax components showed, that mainly primary alcohols with chain length of 26, 28 and 30 carbons contribute to the enrichment of the total wax load (Fig. 3B - D). *myb41-3* as well as *myb41-4* showed the highest accumulation in primary alcohols, whereas in *myb41-1* and *myb41-2*, both mutants of only R2 domain, this effect was slightly smaller. The main reductase of the biosynthesis of primary alcohols up to 30 carbons is CER4/FAR3 (Rowland et al., 2006). We analyzed *CER4/FAR3* gene expression by qRT-PCR analysis. Expression rates of *CER4/FAR3* are 30 times higher for *myb41-1*, 80 times for *myb41-2*, 130 times for *myb41-3* and 110 times for *myb41-4* (Fig. 4A). Moreover, the expression of other genes of the wax biosynthesis pathway like *CER2*, *CER3* and *MAH1* was up to 6-fold higher in the *myb41* lines (Fig. 4B - D). Therefore, we concluded that MYB41 is a negative regulator of *CER4* expression and consequently of the biosynthesis of primary alcohols. Nevertheless, differences within *myb41* mutant alleles were observed. In case of primary alcohol formation *myb41-1* and *myb41-2* showed lower increases in expression *CER4* than *myb41-3* and *myb41-4* (Fig. 4A) and a similar effect was observed for the primary alcohol content (Fig. 3B - D). Next, the amount of cutin monomers was investigated in the *myb41* mutant lines, since this polymer also contains primary alcohols. However, the *myb41* lines did not show significant changes in the cutin content in comparison to WT (Supplemental Fig. 3). The main cutin components, 16:0 dicarboxylic acid (DCA), 18:2 DCA and 18:3 ω-hydroxy alcohol showed no changes either.

MYB41 may be a developmental regulator of wax biosynthesis in stem

Since *myb41-(1-4)* mutant alleles produce less wax in inflorescence stems, we aimed to asses if the repression of wax biosynthesis is regulated during plant development or in response to stress. As a model for young tissue, the top part of the stem from the flowers up to the last internode was used, while the bottom part of the stem (from the first internode to the bottom of the stem) represented the aged tissue. *MYB41* is higher expressed in the basal part of the stem than in top part (Fig. 2), while wax biosynthesis is very active in the younger part of the inflorescence stems (Suh et al., 2005). After

wax analysis via GC-FID, the ratio between the wax content in bottom part and content in the upper part was calulated and shown in percentage. In WT plants, the wax amount in both parts was similar (shown as 100 %). In *myb41-(1-4)* mutant alleles a higher wax content in the bottom part of the stem in comparison to upper part was observed. However, significant changes were detected only in *myb41-2* and *myb41-3* lines (Fig. 4). This result suggests that MYB41 is involved in repression of wax biosynthesis in the older part of the stem. Since MYB41 seems to act as a repressor of wax biosynthesis in inflorescence stems, we analyzed the phenotype of the *myb41-(1-4)* mutant alleles under conditions, where the wax biosynthesis is reduced like growth in the dark or in high humidity (Baker, 1974; Go et al., 2014). WT plants and *myb41-(1-4)* mutant alleles, which were challenged by extended dark or were grown in high humidity did not show any differences in the wax amount (Supplemental Fig. 4). This led us conclude, that MYB41 is repressing wax biosynthesis in a development dependent manner. MYB41 seems not to be involved in the regulation of the wax biosynthesis under stress conditions like extended dark or high humidity.

The R2 domain of MYB41 is required for repressing the biosynthesis of primary alcohols.

Wax analyses on the *myb41-(1-4)* mutant alleles showed that mutations in the R2 domain as well as truncations of the protein at the C-terminus lead to a dysfunction of MYB41. *myb41-1* which lacks only 2 amino acids in the R2 domain, has already a chemotype with reduced amounts of primary alcohols, although it still contains the native R3 domain, the complete motif of the 11 MYB subfamily and the conserved Ser^{251}. In order to investigate if the repression of the wax biosynthesis only depends on the R2 domain we overexpressed *MYB41* as full version and as truncated version (MYB41-R2, containing the N-terminal part of the protein with the R2 domain). Moreover, MYB41-R2 was expressed in *myb41-2*, to eliminate the influence of the native protein. Surprisingly, overexpression of both versions led to a reduction of C26 and C28 primary alcohols in the wax of Arabidopsis inflorescence stems, even though the total wax amount remained unchanged (Fig. 6). Therefore, the expression of *CER4/FAR3* in the lines overexpressing only R2 domain was analyzed. As expected, MYB41-R2 can not only complement the induced expression of *CER4/FAR3* in *myb41-2* but also further reduce its expression (Fig. 7A). In addition, MYB41-R2 could

complement the induced expression of *MAH1,* but it did not reduce it in comparison to WT (Fig. 7B). Together, it seems that the native MYB41 protein as well as its truncated version with only the R2 domain can repress wax biosynthesis in inflorescence stems of Arabidopsis.

MYB41 does not regulate wax biosynthesis in leaves and siliques

Since MYB41 is also expressed in other wax-rich organs like leaves and siliques, we analyzed the wax load in these organs in the *myb41-(1-4)* mutant allele collection. No changes in the wax content as well as in the primary alcohol content of mutant in comparison to WT leaves were detected (Supplemental Fig. 5A, B). Next, the amount and composition of the second cuticle component, cutin, was analyzed in the *myb41-(1-4)* mutant allele collection and WT plants. In total cutin content no differences in comparison to WT were detected (Supplemental Fig. 6A), however one cutin compound, 16:0 DCA was significantly enriched in *myb41-1* and *myb41-2* but not in myb41-3 and myb41-4 (Supplemental Fig. 6B). Although *MYB41* is expressed in leaves, we could not reveal its function in this organ. The wax amount in siliques of the *myb41-(1-4)* mutant allele collection were also analyzed and similar to leaves no changes in the amount of wax and primary alcohols were observed (Supplemental Fig. 7A, B). However, by overexpressing native MYB41 or MYB41-R2 in siliques a reduced amount of C28 primary alcohols was observed, however not as much as was observed for inflorescence stems (Supplemental Fig. 8A,B). Therefore, our data suggest, that MYB41 does not play a significant role in wax biosynthesis in leaves and siliques.

DISCUSSION

The cuticle, composed of wax and cutin, is covering all aerial parts of a plant and its biosynthesis is strictly regulated. Apart from positive regulators of wax biosynthesis, there are also negative regulators (DEWAX1 and DEWAX2) (Go et al., 2014; Kim et al., 2018) known. Here we report that one of the R2R3-MYB transcription factors, MYB41, acts as negative regulator of wax biosynthesis, too.

MYB41 is a negative regulator of primary alcohol biosynthesis

Previous reports about MYB41 focused on the functional analysis of lines overexpressing this transcription factor. We however managed to obtain four different

myb41 mutant alleles via CRISPR/Cas9, which allowed us to further analyze its function. One mutant (*myb41-1*) lacks only 2 amino acids in R2 domain, *myb41-2* and *myb41-3* express only short proteins of a truncated R2 domain, while *myb41-4* lacks 9 amino acids outside any functional domain and shows a shift of Ser^{251} to Ser^{242}, which could be important for the functionality since this serine may be phosphorylated (Hoang et al., 2012) (Fig. 1). A significantly higher content of primary alcohols (of 26 to 30 carbons chain length) was detected in all four genotypes of the *myb41-(1-4)* mutant alleles, while only in *myb41-1*, *myb41-3* and *myb41-4* also increased amounts of wax in comparison to WT were observed (Fig. 3). The main enzyme catalyzing the reduction of fatty acids to primary alcohols in Arabidopsis inflorescence stems is CER4/FAR3 (Rowland et al., 2006). The mutant of *CER4/FAR3* was deficient in primary alcohols, with the exception for primary alcohols with 30 carbons, which were still detected in reduced amounts (Rowland et al., 2006). Indeed, the expression of the gene coding for this enzyme was induced in all *myb41-(1-4)* mutant alleles, from 30 to 130 times in comparison to WT (Fig. 4A). Other genes from wax biosynthesis pathway, like *MAH1*, *CER2*, *CER3* were also expressed at higher levels in *myb41-(1-4)* (up to 6 fold. Fig. 4B - D). This may suggest that MYB41 is directly regulating the expression of *CER4/FAR3,* while the induction of other genes involved in wax biosynthesis could be a result of an indirect regulation or reinforcement of the pathway, to push wax biosynthesis, however more data are needed to asses this relation. So far, there was only one transcription factor described which is directly regulating the expression of *CER4/FAR3*, namely MYB94. MYB94 is a positive regulator of wax biosynthesis and belongs to subgroup 1 of the R2R3-MYB transcription factor family (Lee and Suh, 2014). Nevertheless, they both possess the R2 and R3 DNA binding domains and might bind to the same motifs in the *CER4/FAR3* promotor. Another MYB transcription factor involved in the positive regulation of wax biosynthesis is MYB96 being closely related to MYB94 (Seo et al., 2011; Lee et al., 2016). MYB41 seems to be so far the only negative regulator of wax biosynthesis from the MYB transcription factor family identified so far.

MYB41 might developmentally regulate the wax biosynthesis

It is well known that the phytohormone ABA is a main regulator of wax biosynthesis (Cui et al., 2016). It is inducing wax deposition upon drought to limit non-stomatal water loss (Kosma et al., 2009; Seo et al., 2009). Previous reports about MYB41 have shown

that its expression can be induced by ABA, what we also could confirm in our studies (Supplemental Fig. 9) (Cominelli et al., 2008). External application of ABA results in induction of wax biosynthesis, but mainly in alkanes and aldehydes. This treatment did not induce primary alcohol biosynthesis (Kosma et al., 2009). This led us to hypothesize, that ABA prevents the formation of primary alcohols by inducing the expression of *MYB41* (Fig. 8). It is yet not clear, which wax compounds have an influence on the overall properties of the wax. However, it is known that on the abaxial surface of Arabidopsis leaves primary alcohols are the major wax group, where on the adaxial surface alkanes are more abundant (Buschhaus and Jetter, 2012). Moreover, in *Brassica oleracea*, a higher relative amount of primary alcohols leads to increased resistance towards insects (Eigenbrode and Jetter, 2002). It seems that the synthesis of primary alcohols is tightly regulated during development in inflorescence stems of Arabidopsis. This is also confirmed by the finding, that in *myb41-(1-4)* mutant alleles more wax in lower part of the inflorescence stems in comparison to the upper part was detected (Fig. 5). The basal part of the inflorescence stems is the older and thicker part, while in the top part wax biosynthesis is higher. Furthermore, *MYB41* is more expressed in base part rather than in upper part (Fig. 2). Hence, it may suggest that MYB41 is negatively regulating the biosynthesis of primary alcohols in the aging part of the stem, which is already covered with a thick wax layer and has no need to produce more wax. In contrast to the developmental chemotype no differences in *myb41-(1-4)* mutant alleles upon treatment with high humidity or dark were observed, although it is known that plants produce less wax under these conditions. Therefore, we concluded that the negative regulation of MYB41 is not stress but rather developmentally induced.

In leaves and siliques MYB41 is not involved in primary alcohol biosynthesis suppression

MYB41 is expressed also in leaves and siliques, however we could not observe any significant wax chemotype in those organs. It might be due to the fact, that *CER4/FAR3* expression is not high enough in leaves and siliques in comparison to the situation in inflorescence stems (Rowland et al., 2006). Moreover, it is possible that another fatty acyl-CoA reductase plays a significant role in leaves and siliques. However, we found that one of the cutin monomers – 16:0 DCA – was more abundant in those MYB41 mutants having an impaired R2 domain. Apart from this, we could not reveal a role of MYB41 in leaves and siliques.

R2 domain of MYB41 can alone repress wax biosynthesis

The *myb41* lines obtained described here had mutations in various regions of the *MYB41* gene. It was striking, that *myb41-1,* with only 2 amino acids less in the R2 domain exhibits a phenotype, although it contains no changes in the R3 domain and in Ser^{251} being important for its activation. We therefore hypothesize that the R2 domain is the part of the protein, which plays a crucial role for its proper function. In order to confirm this hypothesis, truncated versions of MYB41 were overexpressed, which contained only the R2 domain and native N-terminal part (MYB41-R2). Indeed we could confirm that the truncated MYB41 protein had the same function as the native MYB41. When both versions were overexpressed in the WT background, a reduction of primary alcohols was observed in Arabidopsis inflorescence stems and a slight reduction in siliques (Supplemental Fig. 8). Moreover, when MYB41-R2 was expressed in *myb41-2,* we could complement the induced expression of *CER4/FAR3,* and observed that this gene is even more reduced in the presence of MYB41-R2 in comparison to WT. This suggests that the expression of the R2 domain of MYB41 only can reduce the biosynthesis of primary alcohols (Fig. 6). However, in one of the mutant alleles, *myb41-4,* the R2 domain is not changed but we can still observe a chemotype. It was shown before that Ser^{251} in MYB41 has to be phosphorylated for proper binding of MYB41 (Hoang et al., 2012). Phosphorylation might change the conformation of transcription factor and therefore allows him to bind to the target sequence or another interactor (Yang et al., 2003). It might be that in *myb41-4*, MYB41 lacks the ability to be phosphorylated, because the Ser^{251} is shifted to the position 242 and thereby MYB41 loses its activity. These results suggest that the C-terminal part of MYB41 is essential for its activation and therefore for proper function, however only the R2 domain is sufficient to repress the expression. The suppression ability of the R2 domain might be due to the fact, that it does not need to be phosphorylated for proper binding to DNA and it might be a physical repressor, since due to its structure it cannot incorporate other proteins and it can block the promotor region for other positive regulators.

The mutant *myb41-2* might be partially functional

The weakest chemotype was observed for *myb41-2,* which was the only mutant with no significantly higher wax load on inflorescence stems. In this mutant, the mutation is located in R2 domain and resulted in a short protein of 47 amino acids. In the R2

domain of the native MYB41 protein, there are three tryptophan moieties forming a tryptophan cluster, which is important for DNA binding activity (Supplemental Fig. 2) (Ogata et al., 1992; Stracke et al., 2001). In *myb41-2,* the second tryptophan (Trp^{37}) is already not present, however two amino acids further there is a $tyrosine^{39}$, which may complement the function of Trp^{37}, because both are aromatic amino acids. Moreover, the open reading frame shift resulted in tryptophan at position 45, therefore in *myb41-2* there are three aromatic amino acids present: Trp^{17}, Tyr^{39} Trp^{45}. This may lead to a partial activity of the R2 domain in *myb41-2,* and as we showed before, just the R2 domain is sufficient to repress the primary alcohol biosynthesis. Moreover, in mutant *myb41-1*, the Trp^{37} is one of the two missing amino acids in the MYB41 sequence and in *myb41-3* the protein do not contain the third tryptophan (Trp^{57}). Along this line in *myb41-1* and *myb41-3,* a stronger phenotype was observed, providing evidence that presence of three aromatic amino acids in the R2 play a major role in MYB41 activity.

CONCLUSIONS

Previously it was shown that MYB41 could induce suberin-like lamellar structures in Arabidopsis leaves when it was overexpressed. Within cutin monomers of MYB41 overexpressing lines, suberin-like monomers were more abundant. This may suggest that MYB41 is a positive regulator of suberin biosynthesis in roots. Besides, we could also observe that MYB41 is highly expressed in roots And our newly isolated *myb41-(1-4)* mutant alleles, might help to analyze its function in suberin biosynthesis in roots now. It might be that MYB41 plays a dual role: inducing suberin biosynthesis in roots and reducing wax biosynthesis in inflorescence stems, however more research is needed to fully understand its role.

In summary the transcript and chemotype analyses of the collection of *myb41-(1-4)* mutant alleles obtained via the CRISPR/Cas9 approach, provided strong evidence that MYB41 is a negative regulator of wax biosynthesis in Arabidopsis stems. MYB41 is reducing the expression of *CER4/FAR3*, an enzyme responsible for the formation of primary alcohols. Furthermore, the R2 DNA binding domain is sufficient to repress the biosynthesis of primary alcohols.

FIGURE LEGENDS

Figure 1 – Protein structure of MYB41 in its native form and in the four *myb41-(1-4)* mutant alleles. A) MYB41 is composed of two the DNA binding domains R2 and R3. Ser^{251} is a phosphorylation site and essential for its activation. B) Protein structure of mutated MYB41 expressed in *myb41-1, myb41-2, myb41-3, myb41-4*. In *myb41-1* the protein lacks 2 amino acids in the R2 domain only. In *myb41-2* and *myb41-3* the protein is mutated in the R2 domain. It results in a premature stop codon and shortens the protein to 47 or 60 amino acids, respectively. Deletion of 9 amino acids at the C-terminal part of MYB41 was observed in *myb41-4.*

Figure 2 – *MYB41* is expressed in all organs in Arabidopsis. Analysis of 5-week-old plants was performed by qRT-PCR. *ELONGATION FACTOR1* was used as reference gene. Data represent the mean ± STD of three biological replicates.

Figure 3 – Wax load in stems of *myb41-(1-4)* mutant alleles is higher in comparison to WT. A) The total wax load was determined in WT, *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4*. Quantification of primary alcohols with B) 26 carbons C) 28 carbons and D) 30 carbons. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's-t test (*, $P<0.05$).

Figure 4 – Expression of genes involved in wax biosynthesis is induced in stems of *myb41-(1-4)* mutant alleles. Relative expression of A) *CER4/FAR3*, coding for the main fatty acid reductase in Arabidopsis stems. B) *CER2,* which encodes an enzyme from fatty acid elongation part. C and D) *MAH1* and *CER3*, coding for enzymes from the alkane-forming pathway of wax biosynthesis. Analysis of 5-week-old plants was performed via qRT-PCR. Data represent the mean ± SEM of three biological replicates. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, $P<0.05$).

Figure 5 – Wax content of 4-week-old Arabidopsis plants differ between segments of stems in *myb41-(1-4)* mutant alleles. The wax load from the upper part of the stem as well as from the bottom part were analyzed and the ratio between the bottom part wax and the upper part wax was calculated and shown in percentage. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, $P<0.05$).

Figure 6 – Overexpression of the native MYB41 protein and a truncated version (MYB41-R2) leads to a reduction of wax amount in 5-week-old Arabidopsis stems. A) The total wax load was determined in WT, MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 (expressed in *myb41-2*) as well as in MYB41-OX1 and MYB41-OX2 (expressed in WT background). The contribution of primary alcohols with B) 26 carbons C) 28 carbons and D) 30 carbons to the total wax load (%) is shown for overexpression of the indicated lines in the *myb41-2* and the WT background. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Figure 7 – MYB41-R2 truncated protein complements *myb41-2* in Arabidopsis stems. Expression analysis in WT, *myb41-2,* MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 expressed in *myb41-2*), A and B) Expression of *CER4/FAR3* and *MAH1*. Analysis was performed via qRT-PCR. *ELONGATION FACTOR1* was used as reference gene. Data represent the mean ± STD of three biological replicates. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Figure 8 –Model of MYB41 function. ABA is inducing expression of *MYB41,* which leads to repression of *CER4/FAR3*, coding the main fatty acid reductase in Arabidopsis stems. This repression leads to reduce primary alcohol content in wax of Arabidopsis stems.

Supplemental figures

Supplementary Figure 1 – *MYB41* gene sequence alignment from sequences cloned form WT, *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4.* Alignment was performed with ApE plasmid editor (http://jorgensen.biology.utah.edu/wayned/ape/). Red boxes indicate a nucleotide deletion, blue boxes an insertion and green boxes are showing a mismatch.

Supplementary Figure 2 – Protein sequence alignment of the native MYB41 and the mutated versions expressed in *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4.* The ExPASy translate tool of the Bioinformatics Resource Portal was used for obtaining protein sequences (http://web.expasy.org/translate/) and Clustal Omega for protein sequence alignments (http://www.ebi.ac.uk/).

Supplementary Figure 3 – The amount of cutin monomers was not changed in stems of 4-week-old *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4* mutant alleles. Quantification of the A) total amount of cutin monomers. B) Three most abundant cutin monomers: 16:0 dicarboxylic acid (DCA), 18:2 DCA and 18:3 ω-hydroxy fatty acid (18:3 ω-OH). C) Compound classes found in cutin of Arabidopsis stems. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Supplementary Figure 4 – Stress treatment did not change the wax content in stems of *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4* mutant alleles. A and B) For high humidity treatment, 4-week- old plants were grown for 1 week with a humidity of approx. 85 %. The total wax load (A) as well as the amount of primary alcohols were determined (B). C and D) for extended dark treatment, 4-week-old Arabidopsis plants were kept in the darkness for 5 days. The total wax load (C) as well as the amount of primary alcohols were determined (D). Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments.

Supplementary Figure 5 – Wax on the leaf surface was not changed in *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4* mutant alleles of 4-week-old Arabidopsis plants. A) total wax load. B) Primary alcohol incorporation into wax. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments.

Supplementary Figure 6 – The amount of cutin monomers was not changed in leaves of 4-week-old *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4* mutant alleles. Quantification of the A) total amount of cutin monomers. B) Three most abundant cutin monomers: 16:0 dicarboxylic acid (DCA), 18:2 DCA and 18:3 ω-hydroxy fatty acid (18:3 ω-OH). C) Compound classes found in cutin of Arabidopsis leaves. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments.. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Supplementary Figure 7 – The amount of the wax load was not changed in siliques of 5-week-old *myb41-1*, *myb41-2*, *myb41-3* and *myb41-4* mutant alleles. A) Total wax load, B) primary alcohol incorporation in wax. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments.

Supplementary Figure 8 – Overexpression of the native MYB41 protein and a truncated version (MYB41-R2) does not lead to reduction of wax in 5-week-old Arabidopsis siliques. A) The total wax load was determined in WT, MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 (expressed in *myb41-2*) as well as in MYB41-OX1

and MYB41-OX2 (expressed in WT background). The contribution of primary alcohols with B) 26 carbons C) 28 carbons and D) 30 carbons to the total wax load (%) is shown for the overexpressing lines of the lines indicated in the *myb41-2* and the WT background. Analysis was performed by GC-FID. Data represent the mean ± STD of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Supplementary Figure 9 – Influence of ABA on *MYB41* expression. 8-day-old seedlings were treated with 10 μM ABA diluted in water:methanol (90:10, v:v) or mock water:methanol (90:10, v:v). Analysis was performed via qRT-PCR. Data represent the mean ± STD of three biological replicates. *ELONGATION FACTOR1* was used as reference gene. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

Supplemental Table 1 – Primers used in the study.

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LITERATURE

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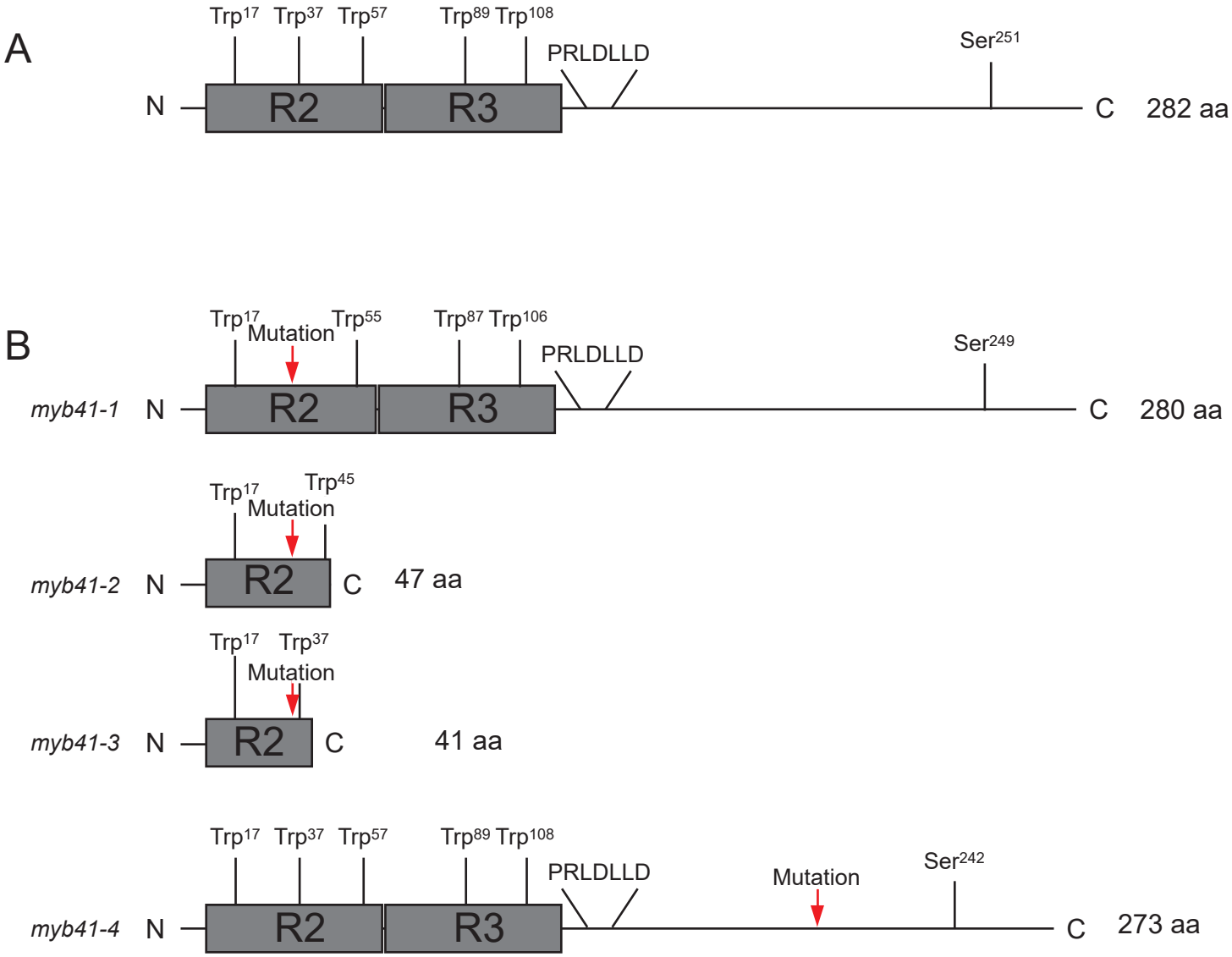


Figure 1 – Protein structure of MYB41 in its native form and in the four *myb41-(1-4)* mutant alleles. A) MYB41 is composed of two the DNA binding domains R2 and R3. Ser^{251} essential is a phosphorylation site and essential for its activation. B) Protein structure of mutated MYB41 expressed in *myb41-1*, *myb41-2, myb41-3, myb41-4*. In *myb41-1* the protein lacks 2 amino acids in the R2 domain only. In *myb41-2* and *myb41-3* the protein is mutated in the R2 domain. It results in premature stop codon and shortening the protein to 47 or 60 amino acids, respectively. Deletion of 9 amino acids at the C-terminal part of MYB41 was observed in *myb41-4.*

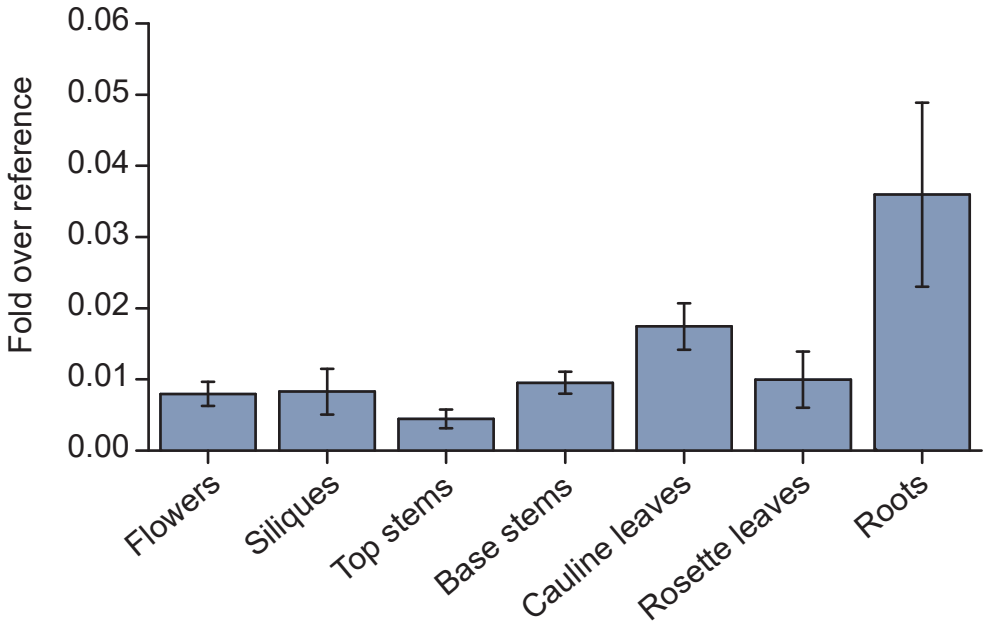


Figure 2 – *MYB41* is expressed in all organs in Arabidopsis. Analysis of 4-week-old plants was performed by qRT-PCR. *ELONGATION FACTOR1* was used as reference gene. Data represent three biological replicates.

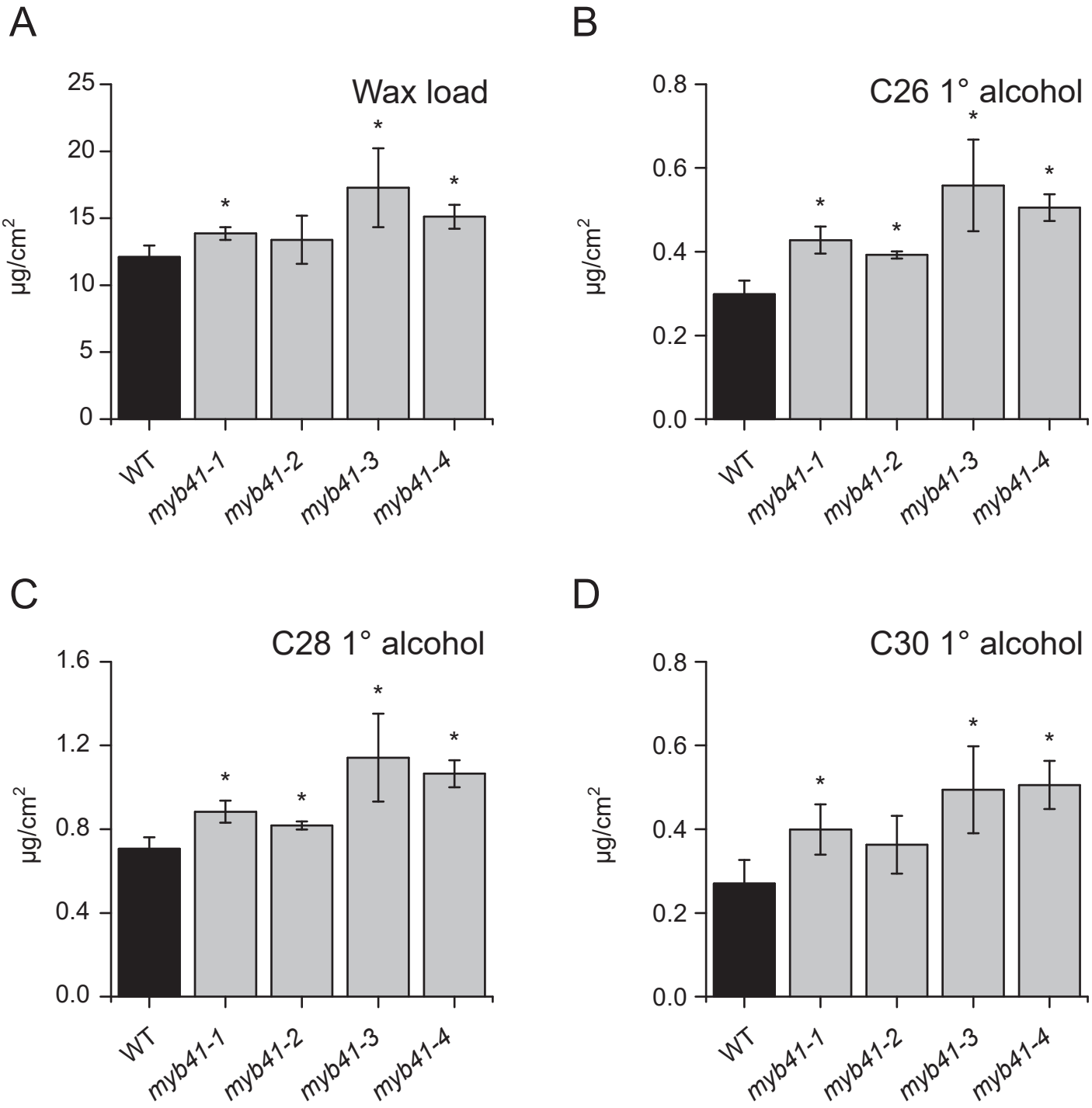


Figure 3 - Wax biosynthesis in stems of *myb41-(1-4)* mutant alleles is higher in comparison to WT. A) The total wax load were determined in WT, *myb41-1, myb41-2, myb41-3* and *myb41-4* Quantification of primary alcohol with B) 26 carbons C) 28 carbons and D) 30 carbons. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's-t test (*, P<0.05).

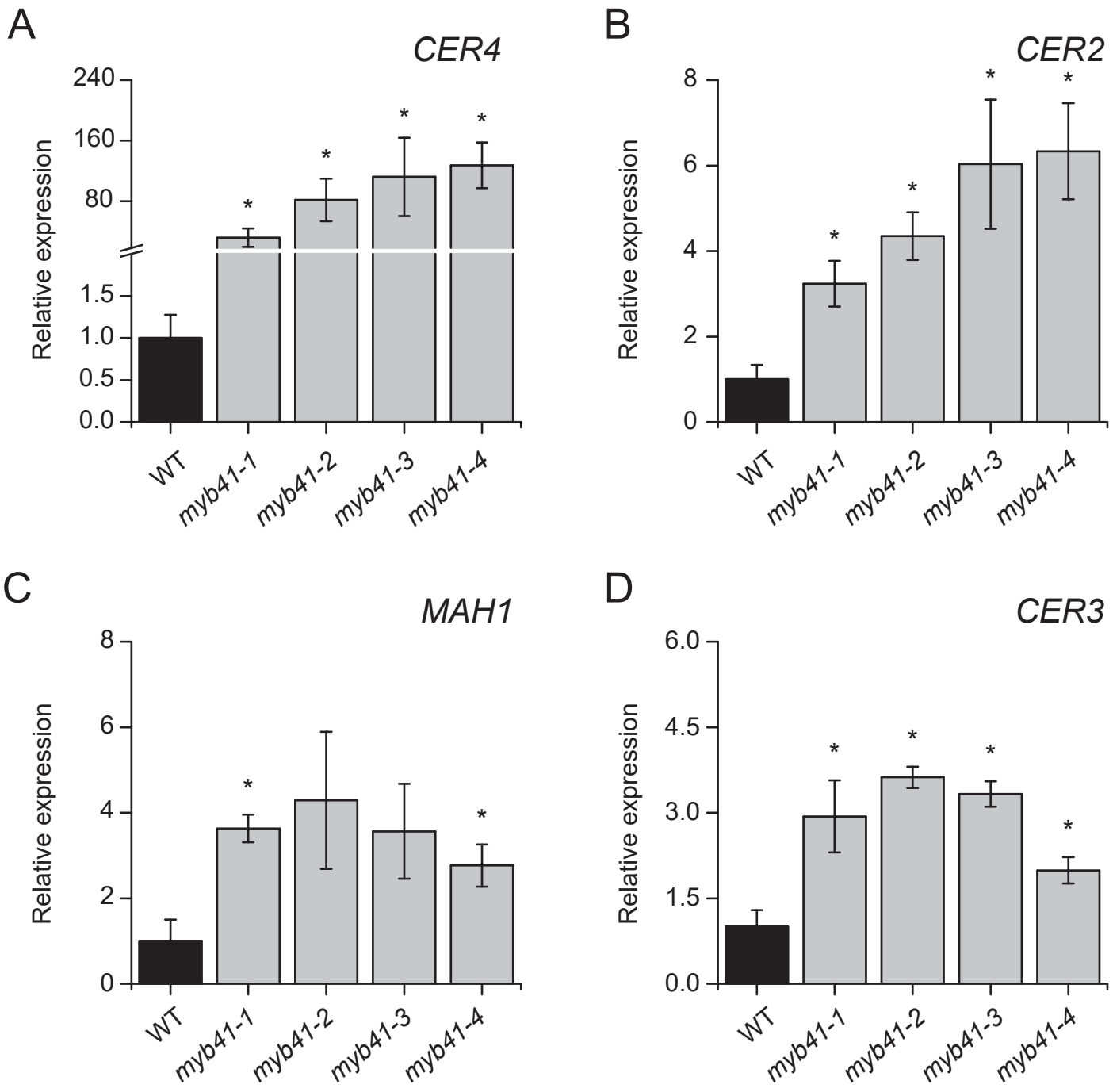


Figure 4 – Expression of genes involved in wax biosynthesis is induced in stems of *myb41-(1-4)* mutant alleles. Relative expression of A) *CER4/FAR3*, coding main fatty acid reductase in Arabidopsis stems. B) *CER2*, which encodes an enzyme from fatty acid elongation part. C and D) *MAH1* and *CER3,* coding enzyme from alkane forming pathway of wax biosynthesis. Analysis of 5-week-old plants was performed via qRT-PCR. Data represent three biological replicates. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

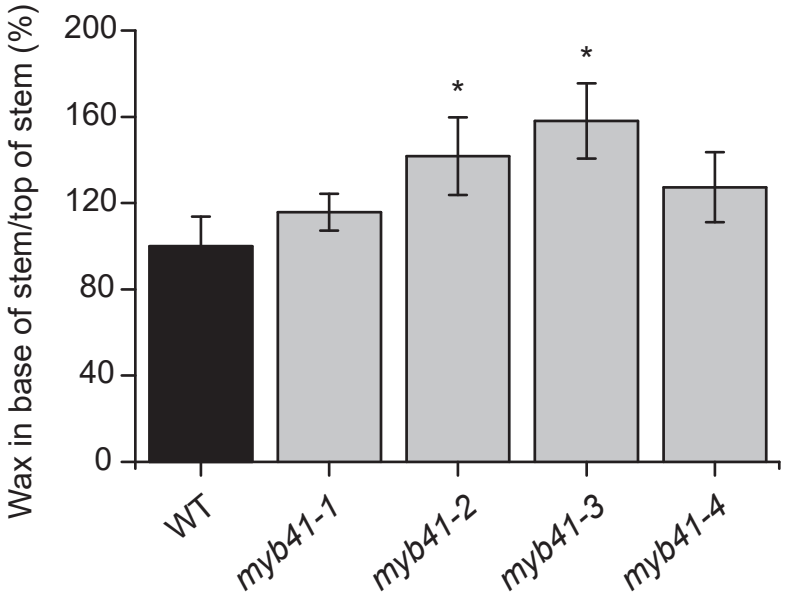


Figure 5 – Wax content of 4-week old Arabidopsis plants differ between segments of stems in *myb41-(1-4)* mutant alleles. The wax load from upper part of stem as well as from bottom part were analyzed and the ratio between bottom part wax/upper part wax was calculated and shown in percentage. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, $P<0.05$).

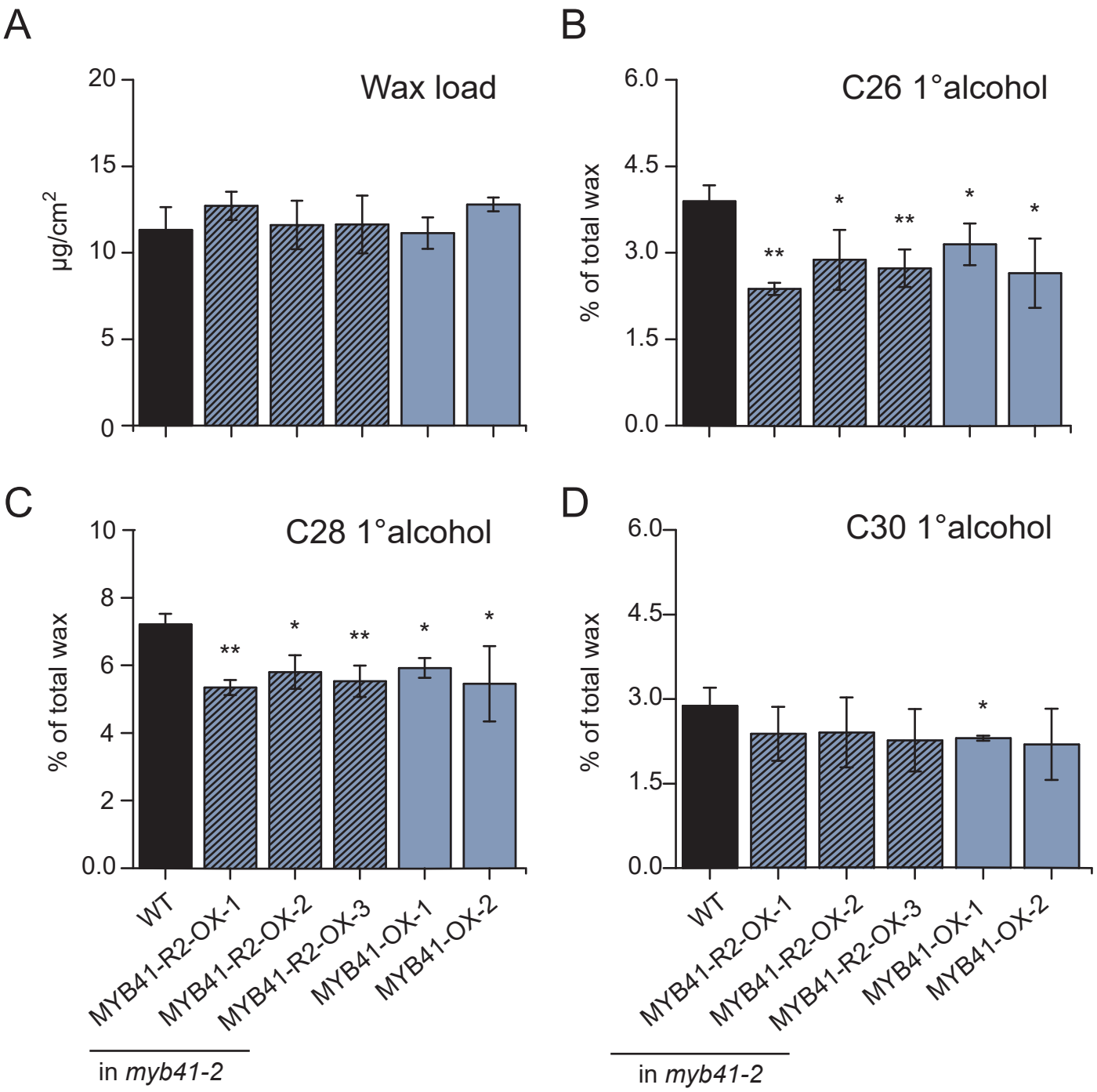


Figure 6 – Overexpression of the native MYB41 protein and a truncated version (MYB41-R2) leads to reduction of wax amount in 5-week old Arabidopsis stems. A) The total wax load were determined in WT, MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 (expressed in *myb41-2*) as well as in MYB41-OX1 and MYB41-OX2 (expressed in WT background). The contribution of primary alcohols with B) 26 carbons C) 28 carbons and D) 30 carbons to the total wax load (%) is shown for overexpression of the indicated lines in the *myb41-2* and the WT background. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

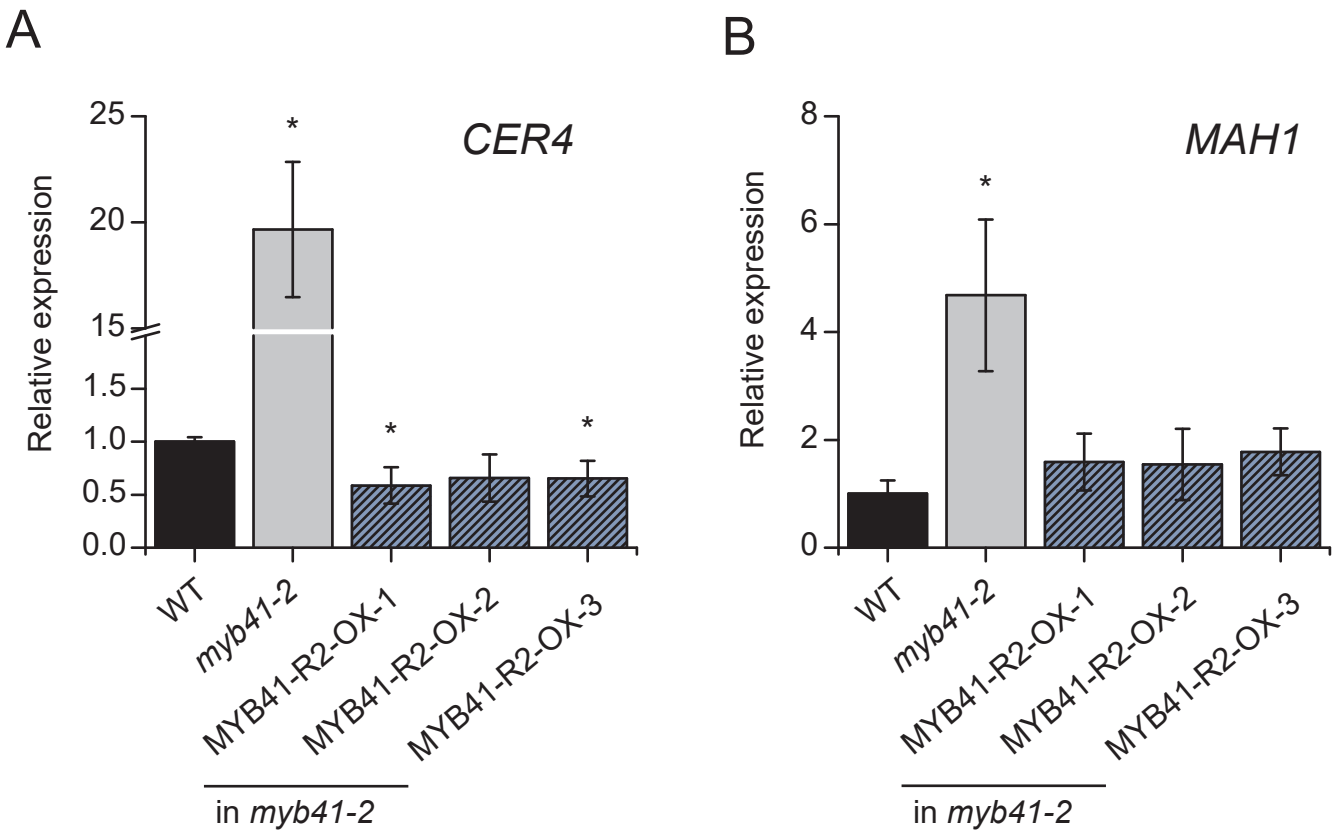


Figure 7 – MYB41-R2 truncated protein complements *myb41-2* in Arabidopsis stems. Expression analysis in WT, *myb41-2,* MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 expressed in myb41-2), A) and B) Expression of *CER4/FAR3* and *MAH1*. Analysis was performed via qRT-PCR. *ELONGATION FACTOR1* was used as reference gene. Data represent three biological replicates. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).

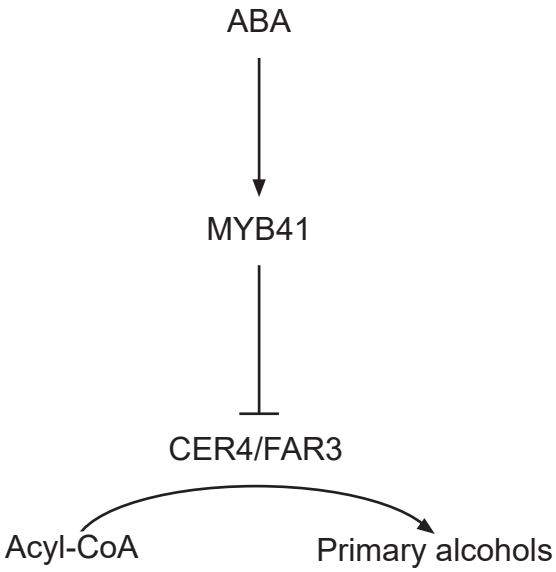
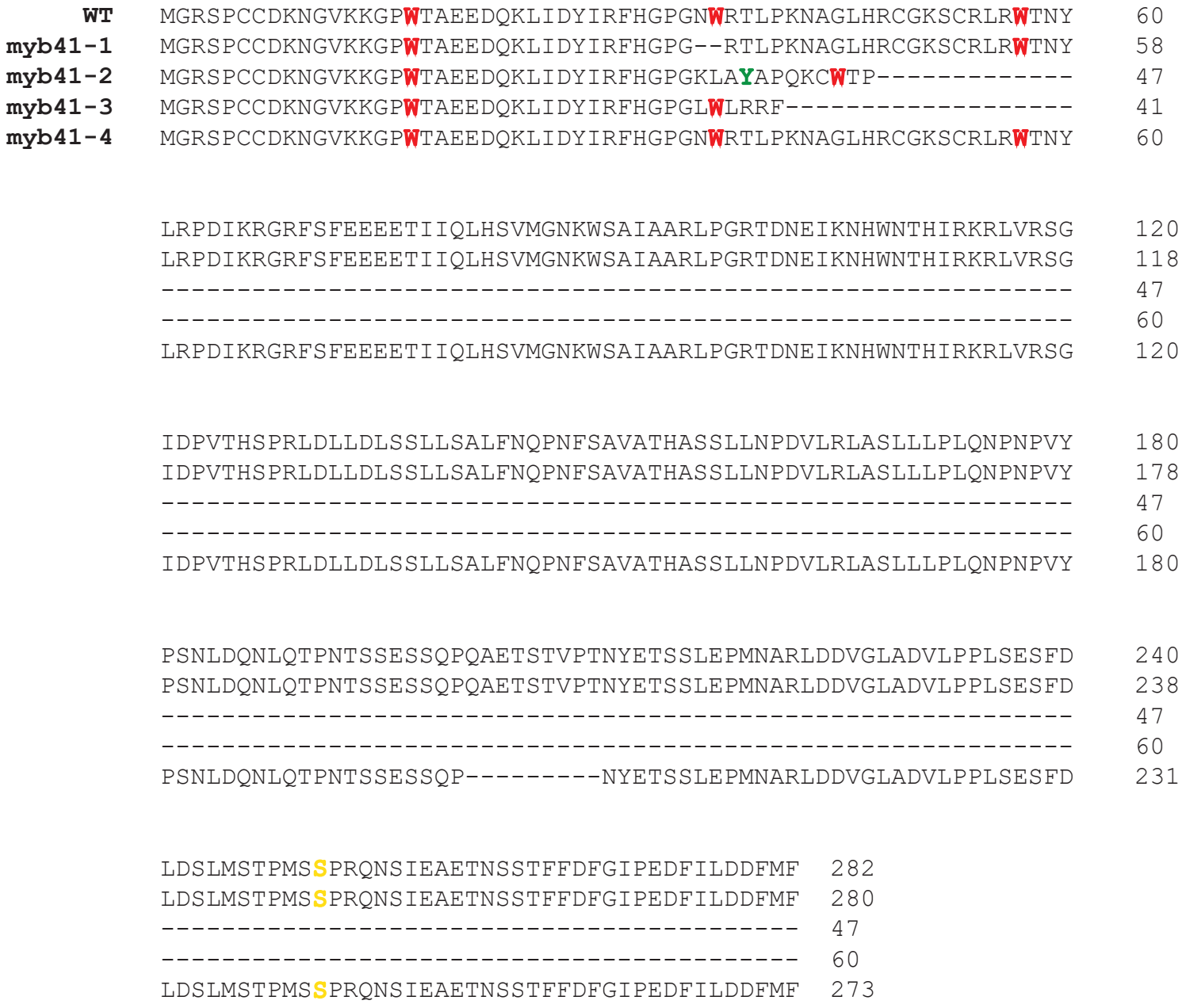
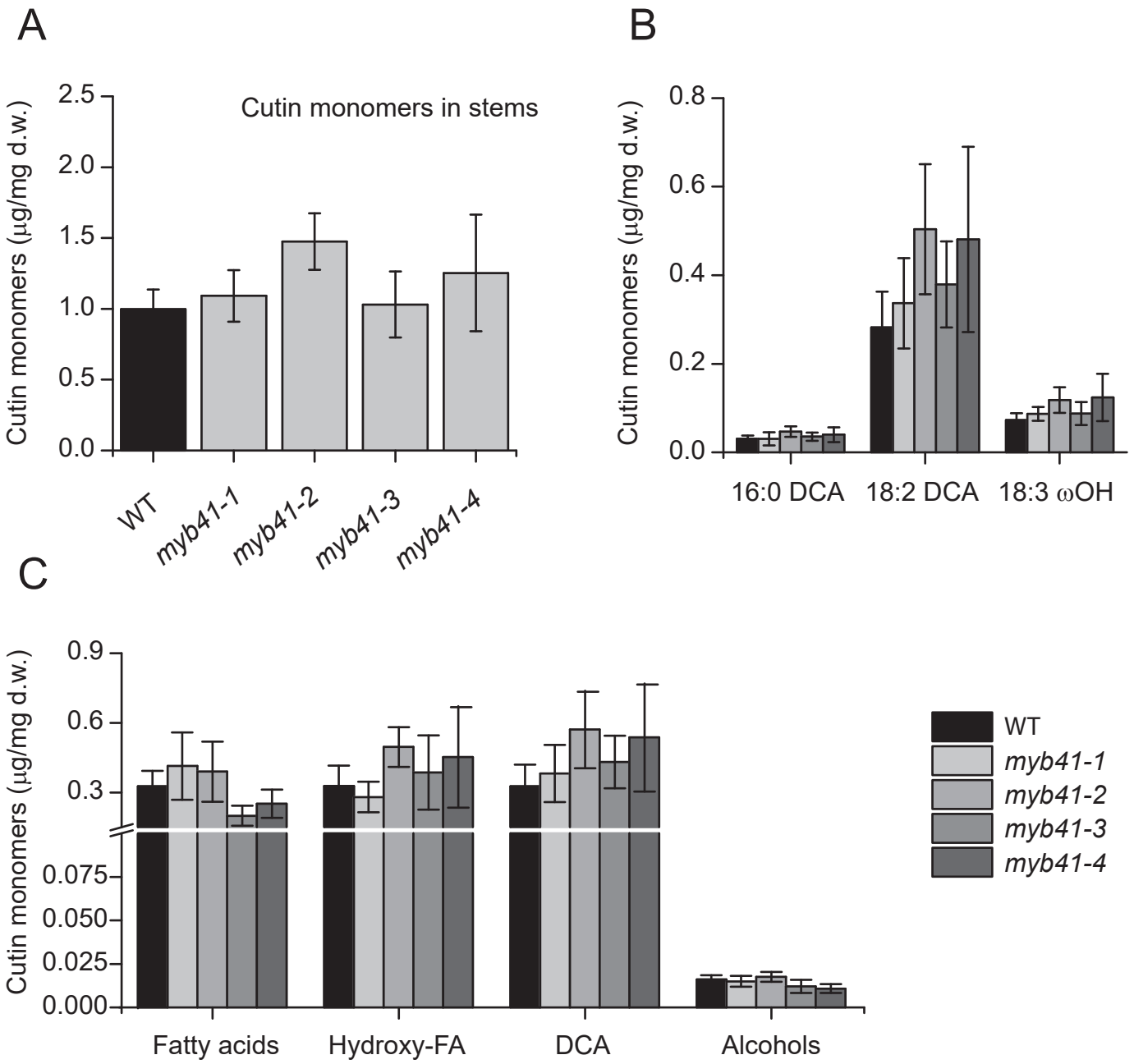


Figure 8 – Model of MYB41 function. ABA is inducing expression of *MYB41*, which leads to repression of *CER4/-FAR3*, coding the main fatty acyl-CoA reductase in Arabidopsis stems. This repression leads to reduce primary alcohol content in wax of Arabidopsis stems.

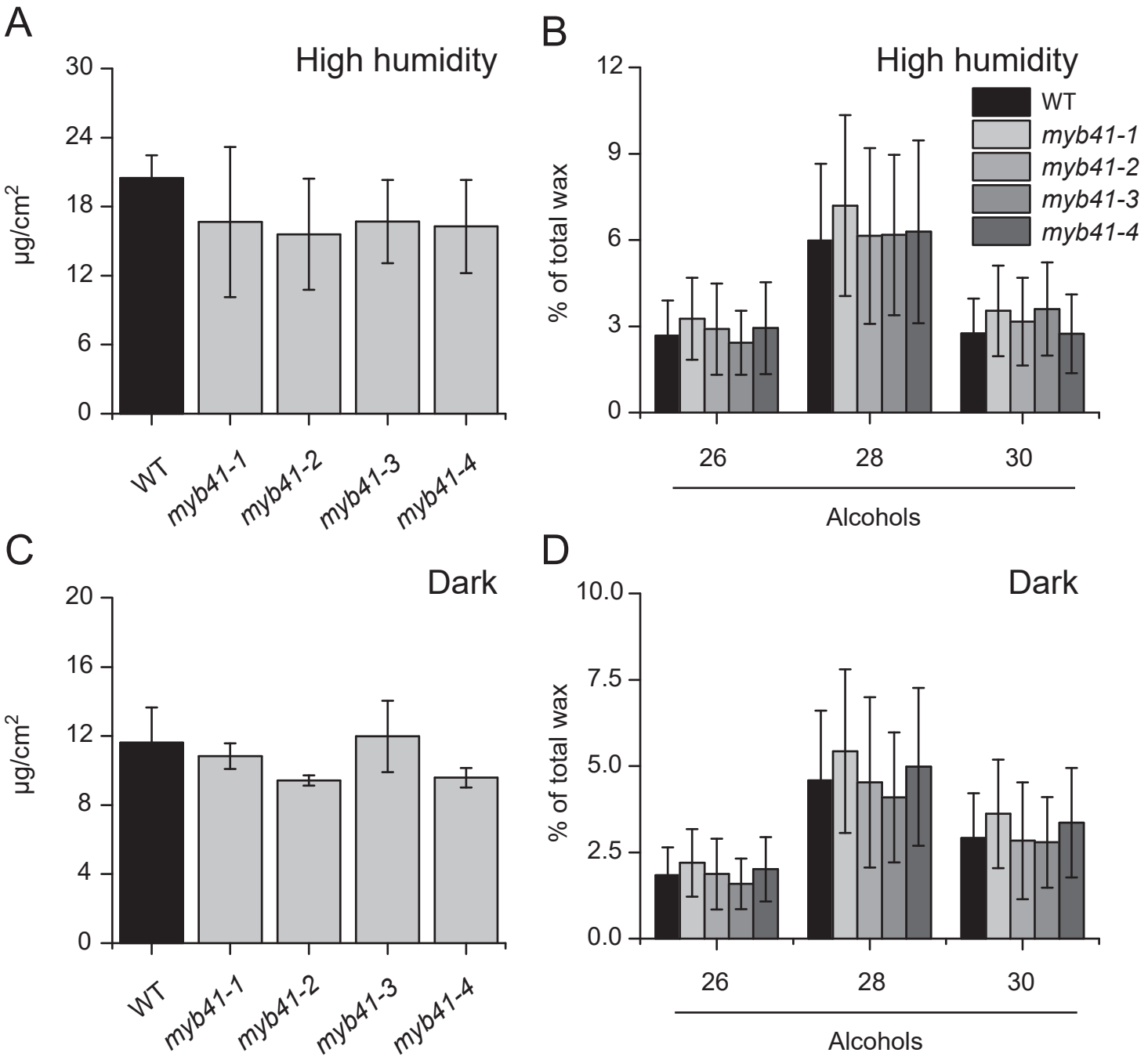
Supplementary Figure 1 – *MYB41* gene sequence alignment from sequences cloned from WT, *myb41-1, myb41-2, myb41-3* and *myb41-4*. Alignment was performed with ApE plasmid editor (http://jorgensen.biology.utah.edu/wayned/ape/). Red boxes indicate a nucleotide deletion, blue boxes an insertion and green boxes are showing a mismatch.



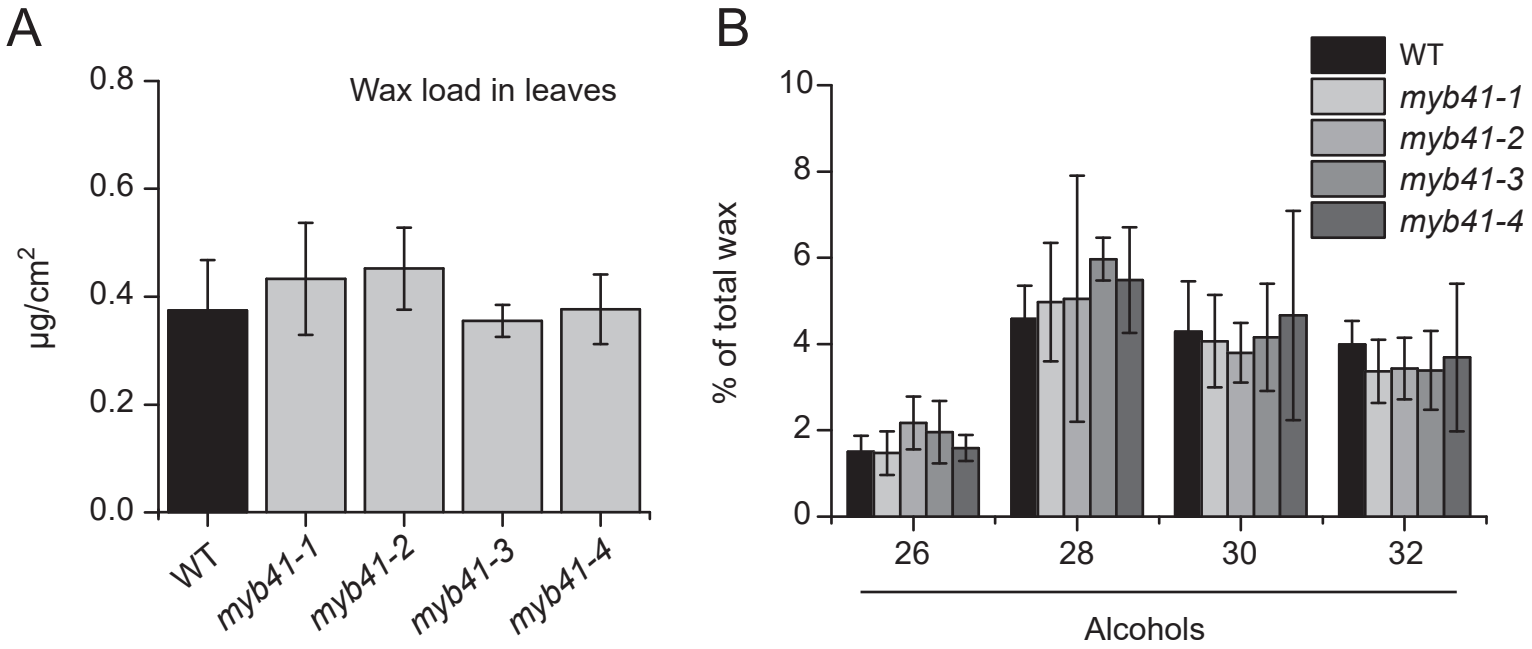
Supplementary Figure 2 – Protein sequence alignment of a native form and in mutated versions in *myb41-1, myb41-2, myb41-3* and *myb41-4*. The ExPASy translate tool of the Bioinformatics Resource Portal was used for obtaining protein sequences (http://web.expasy.org/translate/) and Clustal Omega for protein sequence alignments (http://www.ebi.ac.uk/).



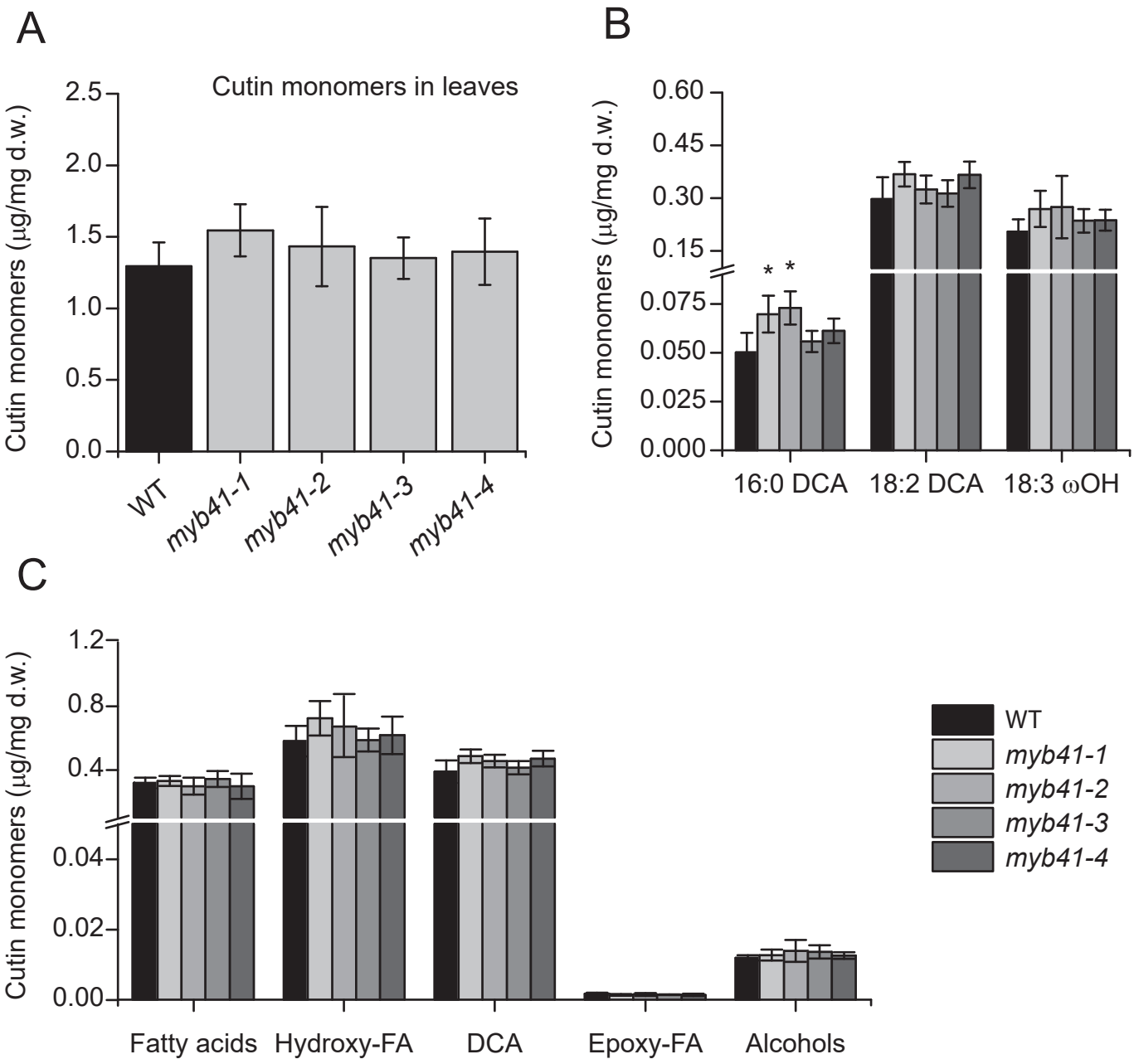
Supplementary Figure 3 – The amount of cutin monomers was not changed in stems of 4-week old *myb41-1, myb41-2, myb41-3* and *myb41-4* mutant alleles. Quantification of the A) total amount of cutin monomers. B) three most abundant cutin monomers: 16:0 dicarboxylic acid (DCA), 18:2 DCA and 18:3 ω-hydroxy fatty acid (18:3 ω-OH). C) compound classes found in cutin of Arabidopsis stems. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).



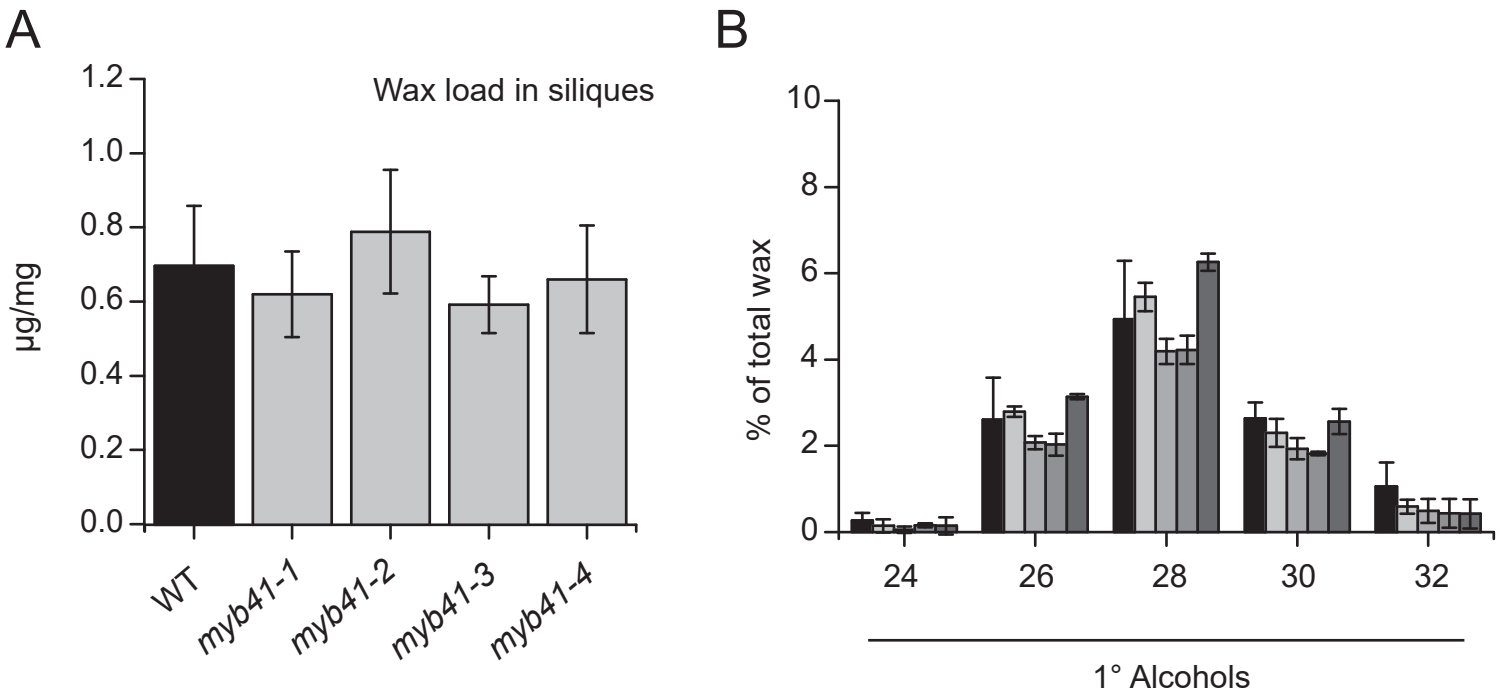
Supplementary Figure 4 – Stress treatment did not change the wax content in stems of *myb41-1, myb41-2, myb41-3* and *myb41-4* mutant alleles. A and B) For high humidity treatment, 4-week old plants were grown for 1 week with humidity of approx. 85 %. The total wax load (A) as well as the amount of primary alcohols were determined (B). C and D) for extended dark treatment, 4-week old Arabidopsis plants were kept in the darkness for 5 days. The total wax load (C) as well as the amount of primary alcohols were determined (D). Analysis was performed by GC-FID. Data represent samples of three independent experiments.



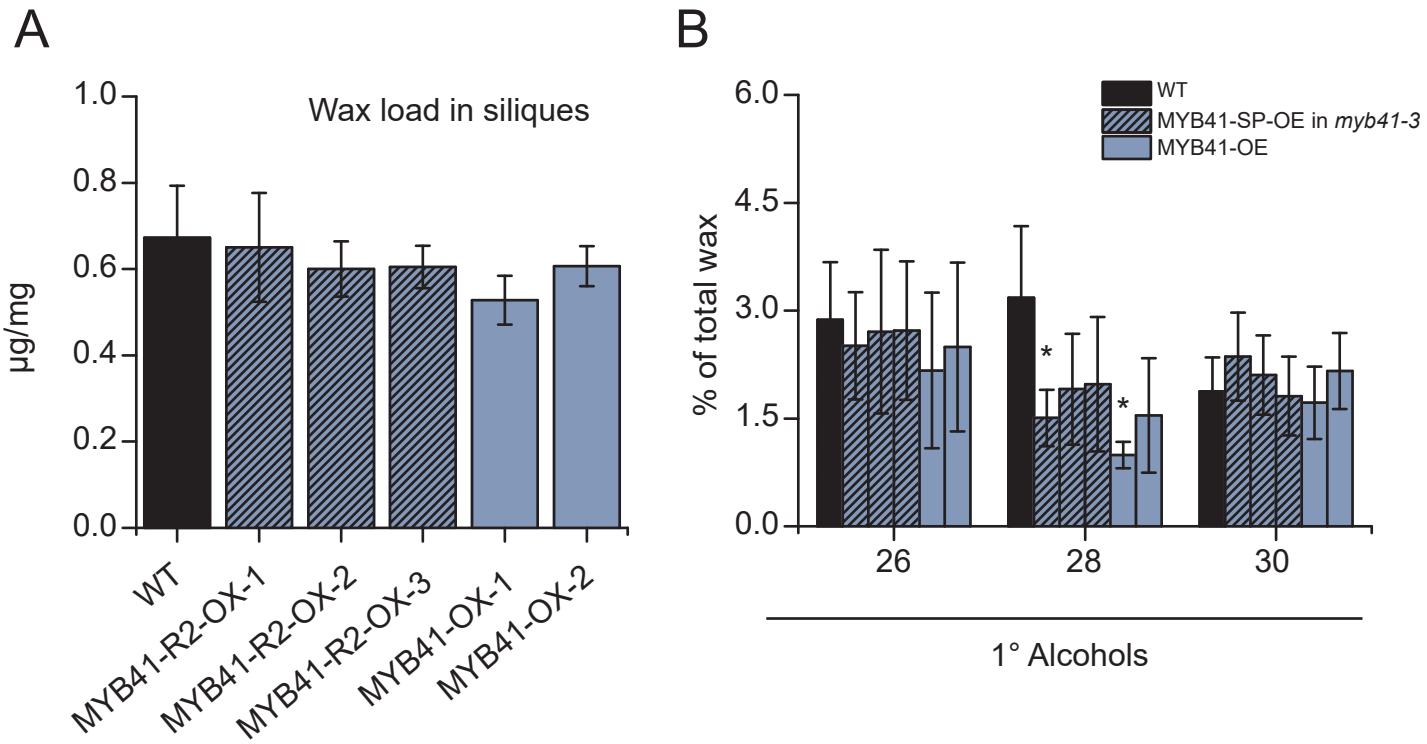
Supplementary Figure 5 - Wax on leaves surface was not changed in *myb41-1, myb41-2, myb41-3* and *myb41-4* mutant alleles of 4-week old Arabidopsis plants. A) total wax load . B) primary alcohol incorporation in wax. Analysis was performed by GC-FID. Data represent samples of three independent experiments.



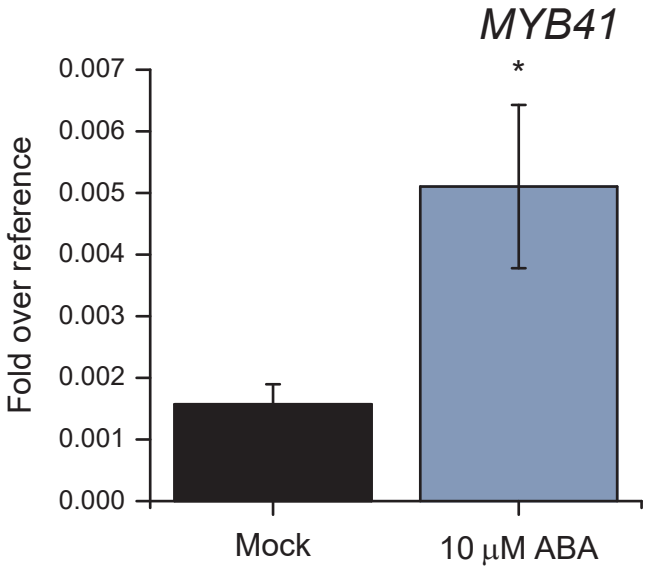
Supplementary Figure 6 - The amount of cutin monomers was not changed in leaves of 4-week old *myb41-1, myb41-2, myb41-3* and *myb41-4* mutant alleles. Quantification of the A) total amount of cutin monomers. B) three most abundant cutin monomers: 16:0 dicarboxylic acid (DCA), 18:2 DCA and 18:3 ω-hydroxy fatty acid (18:3 ω-OH). C) compound classes found in cutin of Arabidopsis leaves. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).



Supplementary Figure 7 – The amount of wax load was not changed in siliques of 5-week old *myb41-1, myb41-2, myb41-3* and *myb41-4* mutant alleles. A) total wax load B) primary alcohol incorporation in wax. Analysis was performed by GC-FID. Data represent samples of three independent experiments.



Supplementary Figure 8 - Overexpression of the native MYB41 protein and a truncated version (MYB41-R2) do not lead to reduction of wax in 5-week old Arabidopsis siliques. A) the total wax load were determined in WT, MYB41-R2-OX1, MYB41-R2-OX2, MYB41-R2-OX3 (expressed in *myb41-2*) as well as in MYB41-OX1 and MYB41-OX2 (expressed in WT background). The contribution of primary alcohols with B) 26 carbons C) 28 carbons and D) 30 carbons to the total wax load (%) is shown for overexpression of the indicated lines in the myb41-2 and the WT background. Analysis was performed by GC-FID. Data represent samples of three independent experiments. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, P<0.05).



Supplementary Figure 9 – ABA influence on *MYB41* expression. 8-day-old seedlings were treated with 10μM ABA diluted in water:methanol (90:10, v:v) or mock water:methanol (90:10, v:v). Analysis was performed via qRT-PCR. Data represent three biological replicates. *ELONGATION FACTOR1* was used as reference gene. Asterisks indicate significant differences from WT by paired sample Student's t-test (*, $P<0.05$).

Supplemental Table 1 – Primers used in the study

Primer name	Accession number	Forward	Reverse
CER2	AT4G24510	GTCTACGATCACGTTCTTGG	CATCATTACGAGCATGAGAGG
CER3	AT5G57800	GGTTAACACCAAGAGAGCAG	CCAACCTCATGATGCTTCC
CER4	AT4G33790	GCTTGTTGATCTCTATCAGCC	GCAGCCCAATAACATGTGT
MAH1	AT1G57750	GCCAGATGTACTTCCAAGC	GCTACCATCTTCATCTGCAA
EF1	AT1G30230	GAGGCAGACTGTTGCAGTCG	TCACTTCGCACCCTTCTTGA
MYB41-SalI -F	AT4G28110	AGCGTCGACACAAAGATGGGAAGATCACCT	
MYB41-BamHI-R	AT4G28110		AGCGGATCCAAGCAAGACATGTGTATGCAAAT
MYB41-R2-BamHI-R	AT4G28110		AGCGAATCCGGTGTGAATGATCAGGCTTA
CRISPR-*MYB41*-BsF	AT4G28110	ATATATGGTCTCGATTGCTGTACTAGTCTCAGCTTGGTT	
CRISPR-*MYB41*-F0	AT4G28110	TGCTGTACTAGTCTCAGCTTGGTTTTAGAGCTAGAAATAGC	
CRISPR-*MYB41*-R0	AT4G28110		AACGGCAATTGGCGTACGCTCCCAATCTCTTAGTCGACTCTAC
CRISPR-*MYB41*-BsR	AT4G28110		ATTATTGGTCTCGAAACGGCAATTGGCGTACGCTCCC

Chapter 4: Discussion

Wax and TAG are essential neutral lipids for plants. Wax is a very hydrophobic mixture of aliphatic compounds and covers all aboveground parts of plants. It seals the plants' surface, limits water loss and is a first barrier against pathogens. TAG is a storage lipid, mostly accumulating in seeds, however it is extensively synthesized in vegetative tissues in response to stress. The main aim of this thesis was to further unravel the regulation of wax and TAG biosynthesis and resolve the role of those compounds in response to wounding.

4.1 TAG is accumulating upon wounding in response to membrane damage

TAG is exhaustively synthesized during seed maturation, to serve as an energy source during germination. Extensive research is focused on the regulation of TAG biosynthesis, mostly in biotechnological purposes of increasing an oil content in the seeds of oil plants like *Brassica napus* or in algae (Vanhercke et al., 2019). Increasing the TAG content, and on that way the amount of oil in a plant is of great economic importance for the supply of industrial products, biofuel and human nutrition (Carlsson et al., 2011). However, usage of plant oils for industrial purposes is highly controversially discussed, since their cultivation competes with food production. Therefore, there are new strategies for increasing TAG content in not reproductive organs of plants, which do not serve as a nutrition source (Vanhercke et al., 2019). However, in vegetative tissues TAG biosynthesis is less active. In leaves, this lipid class represents only c. 0.1 % of the dry weight. Therefore, more research is needed to help to increase the amount of TAG in vegetative tissues like leaves of crop plants. A rate-limiting step of TAG biosynthesis in leaves is the activity of PAH enzymes, which forms DAG by removing the phosphate group from PA and afterwards DAG can be acylated to TAG. The lack of those enzymes leads to accumulation of phospholipids and lower TAG biosynthesis (Craddock et al., 2015). Therefore it was proposed that PA is a central hub for the distribution of precursors to membrane lipids or to TAG biosynthesis (Xu and Shanklin, 2016). However, it is not well understood how this partitioning is regulated. It is known, that the ratio between membrane lipids and TAG is changed during stress conditions, like freezing, ozone

treatment, heat stress, extended dark and wounding (Sakaki et al., 1990; Moellering et al., 2010; Higashi et al., 2015; Vu et al., 2015; Fan et al., 2017). Under all those conditions plants accumulate TAG in leaves. It was shown in chapter 2 (Figure 4a), that upon wounding, plants accumulate 3.5 times more TAG than under normal conditions and that the majority of FA for newly synthesized TAG originated from membrane lipids. This was further supported by the fact that upon wounding the expression of genes downstream from DAG formation was not induced. Furthermore, plastidial lipids, such as DGDG, MGDG or PG are degraded and FA originating from those lipids are incorporated into TAG. This can be analyzed by tracking the incorporation of 16:3 into TAG. Under normal growth conditions 16:3 is not found in TAG in Arabidopsis leaves, but it is known to be very abundant in plastidial lipids, like MGDG and DGDG (Ohlrogge and Browse, 1995). It was shown, that upon wounding and freezing stress SENSITIVE TO FREEZING 2 (SFR2), which belongs to the glycosyl hydrolase family 1 and is localized in plastids, removes galactosyl moieties from MGDG and forms DAG (Moellering et al., 2010; Vu et al., 2015). Mutant plants lacking SFR2 were not able to produce TAG with 16:3 upon wounding and freezing (Moellering et al., 2010; Vu et al., 2015). DAG derived from MGDG by the action of SFR2, can be further acylated to form TAG. This reaction is catalyzed by the main DAG acyltransferases: DGAT1 and PDAT1, since their expression increases upon wounding, as shown in chapter 2 (Figures 3i-j). Furthermore, mutant plants of each of those enzymes show reduced TAG accumulation and both can complement each other's role (Zhang et al., 2009). An intriguing and unresolved question concerning this mechanism is, how this DAG, which is synthesized from MGDG in plastids, is acylated to TAG, since its biosynthesis takes place in ER. One scenario assumes that DAG is exported from the plastids by a yet unknown transporter. Main research is focused on the characterization of the lipid transport from the ER to plastid, hence the DAG transport from plastids to the ER remains elusive and one candidate may be FATTY ACID EXPORT 1 (FAX1) (Li et al., 2015). A second possible scenario is based on a possibility that TAG biosynthesis can take place in plastids. DGAT1 was detected in intact chloroplasts and stroma fractions and it is involved in TAG accumulation, consisting especially of 16:3, during senescence (Kaup et al., 2002). Authors correlated this TAG accumulation with an increased amount and size of plastoglobuli, lipoprotein particles in the stroma of plastids (Kaup et al., 2002). It was suggested that TAG might be produced in plastids and stored there during senescence in plastoglobuli (van Wijk and Kessler, 2017), since

chloroplasts are one of the first organelles showing symptoms of senescence (Kaup et al., 2002). This assumption may also be true for the green alga *Chlamydomonas reinhardtii,* where a starch-less mutant accumulated TAG within plastids (Fan et al., 2011; Goodson et al., 2011). Moreover, another algae, *Phaeodactylum tricornutum,* may also store lipids in plastoglobuli (Balamurugan et al., 2017).

In chapter 2 (Figures S12-S17) it was shown, that upon wounding not only MGDG and DGDG are degraded, but also PG, a phospholipid present in plastidial membranes. One of the possibilities, how PG could be involved in the TAG biosynthesis is its turnover to PA, since those two lipids differ from each other only by a glycerol-moiety attached to the phosphate group. However, it was proven by *in vitro* experiments with [2-^{3}H]-glycerol-labeled PG, that the heavy atoms from this lipid are not transferred to PA (Sankaran and Wu, 1994). Nevertheless, DAG can be obtained from PG via action of phospholipase C, which cleaves off the glycerol phosphate group (Murata, 1983). In addition, a FA can be removed from PG by the plastidial phospholipase A1. One of them, PLASTID LIPASE1 (PLIP1), was found to contribute to TAG biosynthesis by releasing FA from PG, which were exported afterwards out of plastids, most likely by FAX1 for TAG biosynthesis (Wang et al., 2017).

DAG obtained from MGDG, DGDG or PG, needs to be further acylated to form TAG. The two main acyltransferases in Arabidopsis that acylate DAG are DGAT1 and PDAT1 (Katavic et al., 1995; Dahlqvist et al., 2000; Lu and Hills, 2002). There are two more DGAT enzymes present in Arabidopsis - DGAT2 and DGAT3 - however, their activity is much lower than DGAT1 (Zhou et al., 2013; Aymé et al., 2014; Aymé et al., 2018). An Arabidopsis mutant of DGAT1 showed a reduced TAG content in seeds by approx. 70 % (Katavic et al., 1995). Moreover, the TAG content in the leaves of this mutant was also significantly reduced in comparison to WT plants, as shown in chapter 2 (Figure 4a). An acyltransferase, that can take over the function of DGAT1 is PDAT1 (Zhang et al., 2009). The sources of acyl-moieties, which are used as a substrate for the DGAT1 as well as for the PDAT1 catalyzed reaction, differ. DGAT1 is incorporating acyl-CoA from the acyl-CoA pool, whereas PDAT1 is an acyl-CoA independent enzyme, which transfers acyl-moieties directly from phospholipids. Both enzymes however are localized in ER. It was suggested that PDAT1 is responsible for the majority of the TAG production in leaves (Fan et al., 2013). As shown in chapter 2 (Figure 4a), mutant plants of PDAT1 contain a reduced TAG amount in its leaves. It

was shown, that the two main DAG acyltransferases, DGAT1 and PDAT1, have overlapping functions, since TAG accumulation in developing seeds is essential and double mutants in both genes (DGAT1 and PDAT1) are lethal (Zhang et al., 2009). It was shown in chapter 2 (Figure 4a), that single mutants of DGAT1 as well as of PDAT1, are still able to accumulate TAG upon wounding. Nevertheless, the TAG amount is reduced in comparison to WT plants. During the wound response, the two acyltransferases DGAT1 and PDAT1 might replace each other's role in acylating DAG to form TAG.

TAG accumulating PUFA, which are the typical FA of plastidial membranes, was observed for many stress conditions, like wounding or freezing, but also for ozone treatment, heat stress and extended dark. Only one direct actor of this process was found to be SFR2, which is removing galactosyl moieties from MGDG and forms DAG. SFR2 acts in the wounding and freezing response but not during extended dark. However, no master regulator involved in increasing TAG biosynthesis upon stress was found yet. In chapter 2 (Figures 4a, 8, S10-11), TAG accumulation was analyzed in mutants, which are deficient in JA-Ile, the main wound-signal, and reduced in ABA, a stress hormone involved in response to drought. Although, ABA plays an important role in TAG biosynthesis during seed development (Nakashima and Yamaguchi-Shinozaki, 2013), the mutant reduced in ABA was accumulating TAG upon wounding. The same response was observed for the mutant deficient in JA-Ile. These results led to the conclusion that ABA and JA-Ile do not play a severe role in TAG accumulation upon wounding. Most likely, TAG accumulation does not occur in response to wounding *per se*. During wounding, many cells are damaged and lose their integrity, therefore the constituents of the membranes within the cells have to be stored or degraded. Membrane rearrangement takes place also during freezing stress, where non-bilayer forming lipids (like hexagonal structures) occur in membranes (Uemura et al., 1995) and during heat stress, where the portion of unsaturated FA has to be decreased (Higashi et al., 2015). Membrane lipids, rich sources of carbon, are also degraded under conditions of extended dark, when plants suffer from carbon starvation (Fan et al., 2017). Finally, upon ozone treatment, where membranes lose integrity and show reduced permeability, membrane remodeling takes place (Iriti and Faoro, 2009). It seems that all those stress treatments cause membrane readjustment, which leads to TAG accumulation. All of these rearrangements require changes in the composition of membrane lipids. FA derived from membrane lipids cannot just be removed

and degraded in the compartments, where they are released, since they would have to be transported to peroxisomes or mitochondria for β-oxidation. Moreover, during stress conditions the amount of reactive oxygen species increases, which causes peroxidation of unsaturated FA (Garg and Manchanda, 2009). It was shown in yeast and mammalian cells that TAG biosynthesis plays a crucial role in detoxifying FA, which can cause lipotoxicity leading to cell dysfunction and eventually death (Listenberger et al., 2003; Kohlwein, 2010). However, the most likely scenario is that FA are stored in TAG, as a transient reserve.

Newly synthesized TAG is stored in cytosolic lipid droplets, lipid rich organelles or may be stored in plastoglobuli, which was suggested for TAG deposition upon ozone treatment, extended dark or senescence. Lipid droplets are usually not very abundant in leaves under normal conditions, however they accumulate upon stress conditions, like extended dark, senescence or wounding (Chapter 2, figure 7) (Brocard et al., 2017; Fan et al., 2017). Moreover, it was shown in chapter 2 (Figure S9) that the leaf specific CALEOSIN3 - a structural protein of the lipid droplet membrane - is highly expressed upon wounding and it was revealed before, that this protein accumulates upon stress like drought and high salinity (Partridge and Murphy, 2009). The newly synthesized TAG stored in lipid droplets can be used further as an energy source, where the FA moieties can be hydrolyzed and exported for β-oxidation. In senescing leaves TAG formed from degraded organelles can be used as an energy source for reproductive organs (Vicentini and Matile, 1993). A similar situation may occur upon wounding, where TAG can be used as an energy source for regeneration of the tissue or as a source of FA for building membrane lipids.

Taking all together, TAG is accumulating upon wounding and the accumulating FA originated from membrane lipids. This process takes place in response to membrane damage caused by wounding. Membranes are rearranged and lipids building them are undergoing modifications to adjust to new conditions. As shown in chapter 2 (Figures 4a, 8, S10-11), this process is not dependent on the two main phytohormones of the wound response– JA-Ile and ABA. Plastidial membrane lipids, like MGDG or PG can form DAG, which can be further acylated by DGAT1 or PDAT1. Whereas lipases can remove FA from plastidial lipids, which can enter the acyl-CoA pool. Newly synthesized TAG is stored in lipid droplets to be further used, for example, for regeneration processes.

Induction of TAG biosynthesis upon stress conditions is used for biodiesel production in algae for many years (Griffiths and Harrison, 2009; Khan et al., 2017). Upon stress, like nitrogen or phosphate deprivation, algae extensively produce TAG and this process is very well studied (Griffiths and Harrison, 2009; Du and Benning, 2016). Therefore, a strategy for stress-induced TAG biosynthesis in vegetative tissues, like leaves, can be another alternative for production of this lipid class. However, it has to be taken into account that TAG obtained upon stress in leaves, contain mostly PUFA which are prone to oxidation and may therefore have a short shelf live.

4.2 Wax is accumulating upon wounding to seal the damaged site

Upon wounding plants have to rearrange their damaged membranes and they need to seal the wounded area. Sealing the wounded site is one of the first steps of the regeneration process. It prevents the tissue from further damages, but also from water loss via the wounded site. The most studied process of sealing the wounded site in plants is callus formation. Callus is an unorganized mass of parenchymal cells and can give a rise to new organs. Recently it was shown, that callus formation upon wounding is negatively regulated by JA-Ile, since mutants of JA-Ile biosynthesis and perception are accumulating more callus at their wounded area (Ikeuchi et al., 2017). Another strategy to seal the damaged site is callose deposition, which is stimulated by SA (Jacobs et al., 2003; Wang et al., 2013). In addition, lipids - like suberin - can seal the wounded site (Kolattukudy, 2001; Domergue et al., 2010). In chapter 2 (Figure 4b), it was shown, that wax is synthesized in response to wounding. Wax is a mixture of aliphatic compounds, like alkanes, alcohols, aldehydes, wax esters, VLCFA and ketones. Together with cutin, they serve as a hydrophobic barrier covering the plants surface. Thus, wax can provide an excellent material for sealing the wounded area. As mentioned, one of the reasons for sealing the damaged area is the protection from losing water. However, this should not be the case for plants growing under conditions of high humidity, since the moisture gradient inside and outside the plant should not differ as much as for plants growing under normal conditions. Moreover, it is known that plants are producing less wax under high humidity conditions, because they do not need to protect themselves from non-stomatal water loss (Baker, 1974). Comparable results are shown in chapter

2 (Figure 5), where Arabidopsis plants grown under high humidity contained less wax on their leaf surface. Furthermore, wax did not accumulate upon wounding under high humidity. It seems that plants do not need to seal their wounded site by enhanced wax production, when the humidity is high. Similar observations were made before, where plants wounded in high humidity conditions showed a higher permeability of the cuticle, which suggests that their wounded area was not sealed properly (L'Haridon et al., 2011). Besides that, the same report showed that upon wounding in high humidity, plants are producing more reactive oxygen species. This was correlated with a higher cuticle permeability and therefore a pronounced penetration of reactive oxygen species via the wounded area (L'Haridon et al., 2011). However, under the opposite humidity conditions, like drought stress, plants are producing more surface wax (Seo et al., 2011), which becomes obvious by dessert plants that are covered with a very thick wax layer (Xu et al., 2009; Roth-Nebelsick et al., 2012). The major regulator of wax biosynthesis upon drought is ABA. During drought, ABA accumulates, which leads to an increased wax biosynthesis (Seo et al., 2011; Cui et al., 2016). As shown in chapter 2 (Figure 4b), this wax accumulation also takes place upon wounding. However, in plants wounded in high humidity, ABA content did not change and as a consequence, wax did not accumulate. It was shown likewise in a previous report, that wounding in high humidity does not cause an increase in ABA (L'Haridon et al., 2011). The same effect was observed for inflorescence stems, which upon wounding did not accumulate ABA and wax, respectively (Chapter 2, figure 6). Inflorescence stems show a higher surface wax load under normal growth conditions than leaves and they contain many fibers. Moreover, due to their thickness they might not suffer as severely as leaves from drought caused by wounding. Taking all together, wax biosynthesis and transport to the surface are tightly connected to the drought stress response and it is a part of a plants' desiccation protection. Therefore, upon wounding of leaves, plants are experiencing drought stress, since they lose water through the damaged tissue. As a strategy for regeneration and further damage protection, plants produce callus, callose, suberin and as shown in chapter 2 - wax. This finding is supported by the fact, that under conditions where loosing water via the wounded area is low (e.g. high humidity), plants do not produce wax and therefore do not properly seal the wounded area.

4.3 Biosynthesis of wax upon wounding is dependent on JA-Ile, ABA and MYB96

Wax covers all aerial parts of plants and forms together with cutin a hydrophobic barrier protecting plants from water loss, pathogen attack, UV-radiation and it seals the wounded areas. Higher wax amount leads to higher resistance to pathogens like *Pseudomonas Syringae, Xanthomonas campestris* or insects (Vailleau et al., 2002; Gaume et al., 2004; Seo and Park, 2010). This increased resistance could be caused by a longer penetration time needed for pathogens to enter through the thicker hydrophobic layer (Raffaele et al., 2008; Seo and Park, 2010; L'Haridon et al., 2011). However, as shown in chapter 2, wax can also seal the wounded site. Since wax plays an important function to build a barrier against e.g. pathogens but also seals the wounded area, its biosynthesis has to be tightly controlled. Wax biosynthesis is regulated on the transcriptional level, where transcription factors like WIN1/SHN1, MYB96, MYB94, MYB41, MYB30, DEWAX1 or DEWAX2 can positively or negatively affect an expression of genes from wax biosynthesis. This process is also regulated post-transcriptionally by CER7, which is involved in the regulation of *CER3* expression by the control of post-transcriptional silencing (Hooker et al., 2007; Lam et al., 2012). In addition, post-translational regulation might take place in case of CER9, a RING-type E3 ligase (Lü et al., 2012). However, a master regulator of wax biosynthesis is ABA. It was shown, that ABA application enhances the wax biosynthesis and ABA-signaling components are involved in the regulation of the wax biosynthesis (Kosma et al., 2009; Cui et al., 2016). Furthermore, when the ABA level increases upon drought stress, plants are producing more wax (Seo et al., 2011). It was shown in chapter 2 (Figure 4b) that mutant plants reduced in ABA, *aao3-4,* accumulated less wax on the leaves surface, especially the amount of alkanes was reduced. The opposite effect was shown for plants treated with ABA. After ABA application the wax amount increases, especially in alkanes (Kosma et al., 2009). Nevertheless, as shown in chapter 2 (Figure 4b), plants reduced in ABA still accumulate wax upon wounding but in reduced amounts. This correlates with a low, but still enhanced ABA content in *aao3-4* upon wounding and a lower induction of the expression of some genes involved in wax biosynthesis. Moreover, wax did not accumulate in conditions where ABA was not accumulating, like for plants wounded under high humidity as well as for wounded inflorescence stems (Chapter 2, figures 5-6). These results show that ABA is regulating wax biosynthesis, but not only developmentally but also in response to stress like drought or

wounding. Nevertheless, activated ABA signaling needs to trigger transcription factors and those are direct players in the wound-induced wax biosynthesis. There are three transcription factors acting upstream from ABA. All of them belong to the family of MYB transcription factors, MYB94, MYB96 and MYB41 (Kosma et al., 2014; Lee et al., 2016). As shown in chapter 3, MYB41 appeared to be a negative regulator of wax biosynthesis, thus its role in wound-induced wax accumulation is not likely. However, two other transcription factors are known to be positive regulators of wax biosynthesis. MYB94 and MYB96 are involved in the regulation of the alkane-forming pathway and VLCFA elongation (Lee et al., 2016). In addition, MYB94 is also regulating the synthesis of primary alcohols (Lee and Suh, 2014). As shown in chapter 2 (Figure S18), mutant plants lacking MYB94 are able to accumulate wax upon wounding. Despite that, the wax load was much lower in non-wounded mutant plants in comparison to non-wounded WT plants. Remarkably, *myb96* was not able to accumulate wax upon wounding and the expression of genes involved in wax biosynthesis was not induced in this mutant in comparison to WT. It was observed that MYB94 together with MYB96 are involved in wax biosynthesis upon drought, since in mutant plants lacking either one of the transcription factors or both, the expression of genes involved in wax biosynthesis was not induced in contrast to WT. However, MYB94 had a smaller impact on the increased expression of genes from wax biosynthesis. The wax content of *myb94* upon drought was not analyzed (Lee et al., 2016). As mentioned, the increase of expression of genes involved in wax biosynthesis and in consequence wax content upon drought was observed. Upon long term drought stress the leaf surface wax load increased 4-fold and MYB96 had a strong impact on this accumulation (Seo et al., 2011; Lee et al., 2016). In *myb96* wax accumulated only up to 2.5-fold (instead of 4-fold in WT lines) after drought stress and genes involved in wax biosynthesis were not induced as strong as in WT plants (Seo et al., 2011). Besides, plants overexpressing MYB96 contain a high amount of surface wax on the leaves and inflorescence stems and they are more resistant to *Pseudomonas syringae* (Seo and Park, 2010). In addition, it was recently shown, that MYB96 plays an important role in suppressing the negative regulatory role of ABA signaling. MYB96 is repressing the expression of many Rho GTPases of plants (ROP), which are negative regulators of ABA responses, including drought (Lee and Seo, 2019). This supports the findings shown in chapter 2 (Figures 4b, S6), that in *myb96* the wax accumulation upon wounding is abolished, nevertheless those plants accumulate ABA in the same manner as WT plants.

Moreover, since in *myb96* ABA signaling is reduced as was recently shown by Lee and Seo (2019), it was not striking that those plants show a similar wax phenotype in non-wounded plants as mutants reduced in ABA. Since the wax biosynthesis upon wounding is part of a drought stress response (see chapter 2), it was not surprising that MYB96 is also involved in wax regulation in response to wounding. However, the wound induced wax biosynthesis is much less remarkable. Upon wounding the surface wax amount was increased by 40 %, while upon drought stress plants showed an increased wax accumulation up to 400 %. The reason that MYB94 is not involved in regulating the wound response (Chapter 2, figure S18) might be that this transcription factor is activated only upon strong drought stress. Whereas MYB96, might be activated upon mild drought stress, like wounding, since the wax accumulation in *myb96* was abolished, but upon severe drought it had a relatively small impact on wax biosynthesis. A possible explanation could be that upon mild drought stress, like wounding, MYB96 is inducing wax biosynthesis, while upon strong or long-term drought stress MYB94 becomes more important. This hypothesis is supported by the fact, that MYB96 is involved in suppressing negative regulators of ABA signaling and in consequence making it much stronger. However, more experiments with single and double mutants of MYB96 and MYB94 are needed to fully understand their role in wax accumulation upon drought.

It is well established that ABA is an important factor in wax biosynthesis during plant development and upon stress, however until now the role of JA-Ile in the wound-induced wax accumulation was unknown. JA-Ile is orchestrating the wound response (Wasternack and Hause, 2013). It plays an important role in defense towards insects or necrotrophic pathogens (Pieterse et al., 2009). Nevertheless, the first barrier for pathogens to cross is the cuticle. As mentioned, the thickness of cuticle and its composition influences the resistance to insects, necrotrophic fungi or *Pseudomonas syringae* (Samuels et al., 2008). Since the success of pathogen attacks is connected to the cuticle as well as to the JA-Ile-dependent response, one aim of this thesis was to analyze if JA-Ile is involved in wax biosynthesis upon wounding. As shown in chapter 2 (Figure 4b), under normal conditions the amount of wax does not differ between JA-Ile-deficient plants (*dde2-2*) and WT plants. However, upon wounding in *dde2-2* no wax accumulation was observed in contrast to WT. This result was surprising, since there was so far no evidence that JA-Ile may be involved in wax biosynthesis. At the transcriptional level in *dde2-2*, only one gene involved in wax

biosynthesis was not induced upon wounding in contrast to WT, *CER6*/*KCS6,* that encodes a condensing enzyme involved in the VLCFA elongation (Chapter 1, figure 4). CER6/KCS6 is a rate-limiting enzyme of the FAE and has a dominant role in this process (Millar et al., 1999; Fiebig et al., 2000). The lack of the gene coding this enzyme results in a dramatic wax reduction (Fiebig et al., 2000). Moreover, as shown in the Supplemental Figure 1 in chapter 5 (Supplemental Information), the expression of *CER6* increases upon MeJA treatment. The not induced expression of *CER6* might be the reason why wax accumulation upon wounding was abolished in *dde2-2.* Nevertheless, if JA-Ile is involved in the positive regulation of *CER6* and how this process happens remains elusive. Despite that, JA-Ile had another connection to the VLCFA elongation. It was shown that mutants deficient in JA-Ile biosynthesis (*aos*) or JA-Ile perception (*coi1*) generate more callus upon wounding (Ikeuchi et al., 2017). The authors correlated the inhibition of callus formation by JA-Ile with the blocking of plant growth and cell proliferation by this phytohormone. It is known, that JA-Ile provides a balance between the defense response and plant growth (Koo, 2018). Upon stress conditions, like wounding or pathogen attack, this balance is disrupted and the main focus is put on the defense response (Yang et al., 2012; Koo, 2018). Therefore, with higher JA-Ile amounts, plants are reducing their growth and cell proliferation to reinforce the defense response. During stress, in mutants lacking JA-Ile signaling, the suppression of cell growth does not take place, therefore more callus formation upon wounding was observed (Ikeuchi et al., 2017). However, more callus deposition upon wounding, in cell layers surrounding the pericycle, was also observed for plants lacking one of the components of VLCFA elongation, KCS1, which is another condensing enzyme like CER6/KCC6 (Shang et al., 2016). The authors concluded that VLCFA and their derivatives might suppress the organogenic activity of the pericycle cells. Pericycle cells are located in between the endodermis and phloem. However, wax is synthesized in the epidermis, so the wax components are most likely not involved in callus suppression. Nevertheless those results show that lipid-derivatives might influence callus formation. *Vice versa* conclusion was shown in chapter 2, where in JA-Ile-deficient plants wax does not accumulate upon wounding. In this case, in the absence of JA-Ile, more callus might be formed and therefore the wax accumulation to seal the wounded site was not needed or the wax accumulation was suppressed by factors connected with callus deposition. The opposite situation might take place in WT plants, where in presence of JA-Ile less callus is formed and maybe this formation is

suppressed by wax. Nevertheless, this hypothesis needs to be proven experimentally, where callus deposition and wax biosynthesis would be monitored in parallel. As mentioned, not only wax or callus is formed to seal the wounded area but also callose. Callose is a polysaccharide usually present in the cell wall and it is deposited upon wounding around the damaged area to seal it (Jacobs et al., 2003). It was observed, that callose is formed upon SA treatment and it is dependent on that hormone (Jacobs et al., 2003). It was shown in chapter 2 (Figure S5), that in absence of JA-Ile, the amount of SA increases 5 hpw. In *dde2-2* more callose deposition was observed, which correlated with the increased SA amount. It may suggest that an increased amount of SA promoted callose deposition in JA-Ile deficient plants. The relation between JA-Ile and SA is on focus of many researchers. In some of the cases those two hormones work synergistically e.g. activating the signaling response upon biotrophic pathogen attack, however SA and JA-Ile often work antagonistically (Yamada et al., 2012). The antagonistic action between these two hormones was found e.g. upon *Pseudomonas syringae* treatment where plants were co-treated with SA and JA-Ile abolished the JA-Ile-mediated defense or when plants treated with a SA-inducing pathogen showed suppressed defense from JA-Ile-signaling (Felton and Korth, 2000; Pieterse et al., 2001; Spoel et al., 2007; Van der Does et al., 2013). It was concluded in chapter 2, that enhanced callose deposition upon wounding in the JA-Ile-deficient mutant, might be caused by a higher amount of SA in those plants after wounding, which is a result of the described antagonism between these two hormones. However, to fully understand this interaction an experimental prove is needed, where plants deficient in JA-Ile or SA as well as in both phytohormones are analyzed for callose deposition and phytohormone accumulation after wounding. Taking all together, it was shown in chapter 2 (Figure 4b) for the first time, that the lack of JA-Ile influences wax formation upon wounding. Moreover, other strategies to seal the wound, callus and callose formation, are inhibited by JA-Ile. It could not be excluded, that JA-Ile is involved in the positive regulation of genes involved in wax biosynthesis, like CER6/KCS6. The influence of JA-Ile on callus and callose formation and wax biosynthesis is summarized in Figure 9.

To sum up, wax accumulation upon wounding is dependent on the two hormones, ABA and JA-Ile. ABA regulates genes involved in wax biosynthesis via ABA-signaling, whereas the mode-of-

action for the involvement of JA-Ile in this process is not fully clear yet. MYB96 is the key regulator of wax biosynthesis in response to wounding.

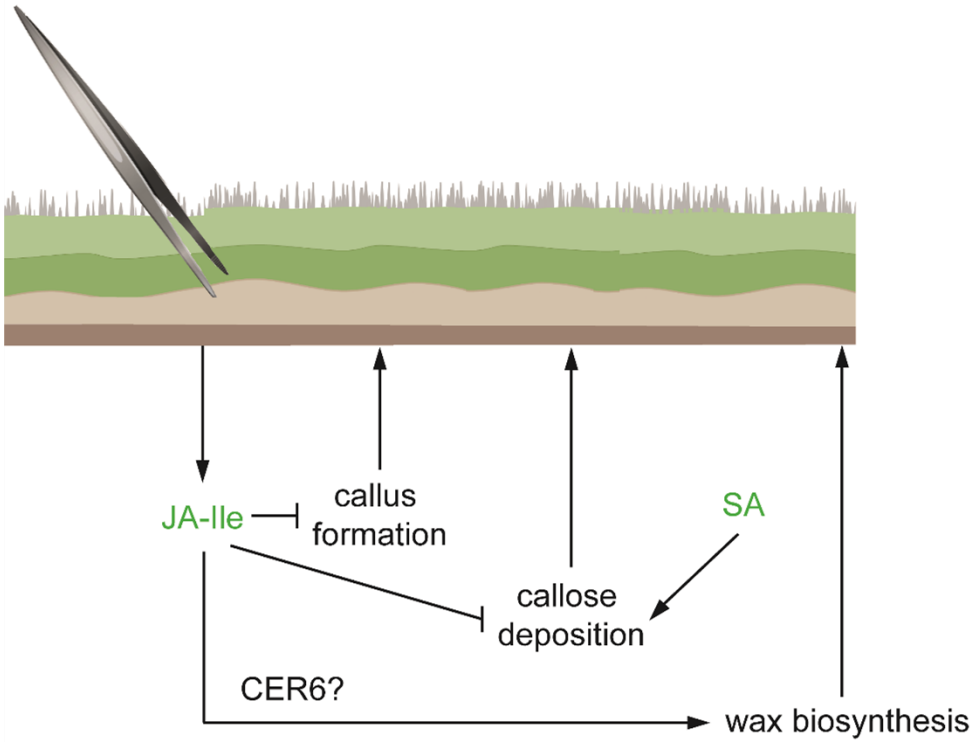


Figure 9. Jasmonoyl-isoleucine is involved in sealing the wounded site.
Mechanical wounding induces the biosynthesis of jasmonoyl-isoleucine (JA-Ile), which is negatively regulating callus formation as well as callose deposition. However, callose biosynthesis is stimulated by an increased amount salicylic acid (SA). In addition, JA-Ile regulates also wax biosynthesis but the direct mechanism of this process is unknown. Nevertheless, JA-Ile might regulate expression of *CER6*, which codes for an enzyme involved in the fatty acid elongation.

4.4 MYB41 negatively regulates wax biosynthesis

Plants adjust their wax content due to the surrounding conditions. During a dark period, the wax biosynthesis is less active since plants do not need to protect themselves from UV-radiation. A similar effect to plants is caused by growth under high humidity conditions, where less wax is produced because of a lowered water loss rate. In addition, in aging tissue the wax biosynthesis is less active. Plants can also adjust their wax composition, however the specific function of the

components is not fully understood. It was shown, that high amounts of primary alcohols on leaves of *B. oleracea* can increase their resistance to insects (Eigenbrode and Jetter, 2002). Furthermore, the wax composition defines the structure of the wax crystals present on the leaves surface. Incorporation of shorter alkanes (less than 29 carbons) results in a change of the shape of the wax crystal to a platelet-like structure (Pascal et al., 2019). However, more biophysical experiments are needed to fully reveal the properties of single wax compounds. The adjustment of wax biosynthesis to changing environmental conditions requires a precise and tight regulation. The strategy of reducing wax biosynthesis is obviously to decrease the expression of genes coding for enzymes involved in this process. It can be achieved by reducing the activity of positive regulators of wax biosynthesis and by stimulating negative regulators. So far, there were two transcription factors, that negatively influence wax biosynthesis, DEWAX1 and DEWAX2. The first is highly expressed in the dark. DEWAX1 diminishes the alkane, VLCFA and primary alcohol biosynthesis, whereas DEWAX2 is specific towards the alkane production (Go et al., 2014; Kim et al., 2018). In chapter 3 (Figures 3, 6) it was shown that MYB41 is another negative regulator of the wax biosynthesis. MYB41 consists of two DNA binding domains, R2 and R3. It belongs to the subfamily 11 of the MYB transcription factors. All of them harbor a highly conserved amino acids motif (PRLDLLD) directly behind the R2R3 domain and a serine at position 251, which needs to be phosphorylated for proper binding of the protein to DNA (Dubos et al., 2010; Hoang et al., 2012). A first study on MYB41 was conducted on *MYB41* overexpression lines. It revealed that these lines showed a higher permeability of the cuticle in leaves (Cominelli et al., 2008). In comparison to WT plants, some of the genes involved in cutin and wax biosynthesis were down-regulated in this line. Moreover, the authors found that *MYB41* is highly expressed upon desiccation, ABA and salt treatment (Cominelli et al., 2008). Another group confirmed these results and found that MYB41 might suppress the expression of genes involved in salt stress response (Lippold et al., 2009). This was a first hint that MYB41 might be a transcriptional repressor. Since the plants overexpressing this transcription factor are exhibiting an impaired cuticle, it could the assumed that MYB41 acts as a repressor of wax and cutin biosynthesis. In chapter 3 (Figures 4, 7) it was shown, that MYB41 is indeed a repressor of wax biosynthesis and it suppresses the expression of *CER4*/*FAR3* and in consequence the primary alcohol biosynthesis is down-regulated. These results were in contradiction with previously published data where MYB41 was introduced as a positive regulator

of wax biosynthesis in Arabidopsis leaves (Kosma et al., 2014). However, in this study the authors based their research on only one *MYB41* overexpression line. This gain-of-function line exhibited a high wax content on the leaf surface. Short primary alcohols, specific for suberin, contributed most to this increase. However, the authors observed a reduction of the amount of primary alcohols with a chain length of 26 and 28 carbons. This finding correlates with the results shown in chapter 3 (Figure 3), where *myb41* mutant alleles had a higher content of those primary alcohols in Arabidopsis inflorescence stems (Kosma et al., 2014). Moreover, Kosma et al. observed that VLCFA with 26 and 28 carbons accumulate in the wax, which might suggest that they are not reduced to primary alcohols. They also concluded that the reduced amount of primary alcohols with a chain length of 26 and 28 carbons is a result of an increased amount of the shorter suberin-like-alcohols, whose synthesis is promoted by the overexpression of *MYB41*. This correlates with another observation, that in the MYB41 gain-of-function lines, suberin-like lamellae accumulated in leaves, which are root specific and are usually not present in leaves. Altogether, it led them to speculate, that MYB41 is a positive regulator of wax and suberin biosynthesis (Kosma et al., 2014). However, the root suberin analysis in the *MYB41* overexpression line was never performed, as well as those finding were never proven by loss-of-function mutants, which for the first time were introduced in chapter 3 of this thesis. Nevertheless, the statement that MYB41 is a positive regulator of wax biosynthesis was repeated in a few publications (Lee and Suh, 2013; Al-Abdallat et al., 2014; Vishwanath et al., 2015). To conclude, data presented in chapter 3 of this thesis provide evidence that MYB41 is a negative regulator of primary alcohols, which are one of the wax components. Moreover, this conclusion is based on the chemotype analysis of four *myb41* mutant alleles as well as on MYB41 overexpression lines.

There may be up to 200 different MYB transcription factors in Arabidopsis. They regulate the primary and secondary metabolism during developmental processes as well as the response to various stresses. They are divided into 25 subfamilies described for MYB transcription factors. This subdivision is established on the basis of conserved amino acid motifs present in each transcription factor (Stracke et al., 2001; Dubos et al., 2010). MYB41 belongs to subfamily 11 and is closely related to MYB74 and MYB102 with 54 % and 63 % amino acid sequence identity, respectively (Stracke et al., 2001). MYB74 is up-regulated upon salt stress and has an unknown function (Xu et al., 2015). Whereas MYB102 is involved in the defense against herbivores and is

induced upon wounding, osmotic stress, salt and ABA treatment (Denekamp and Smeekens, 2003; De Vos et al., 2006; Zhu et al., 2018). MYB41 is also conserved in the plant kingdom. It is present in the bryophyte *Physcomitrella patens*, but also in crop plants like *Brassica napus*, *Camelina sativa* or *Helianthus annuus.* Interestingly, in *P. patens, B. napus* and *C. sativa* the wax is very rich in primary alcohols, whose biosynthesis is negatively regulated by MYB41 in *A.thalina* (Buda et al., 2013; Lee et al., 2014; Liu et al., 2014). Arabidopsis MYB41 shares high similarity to MYB41 present in *P. patens* (70 % identity) and the main wax components in *P. patens* are primary alcohols (Buda et al., 2013; Resemann et al., 2019). The function of MYB41 in this species was not analyzed yet. Due to its wax composition and to the fact that in *P. patens* suberin is not present, it might be an exciting research project to analyze the function of MYB41 in this model organism. It was suggested that MYB41 might play a role in suberin biosynthesis and in *MYB41* overexpression lines a lamellae-like structure, known for suberin in roots, was present in leaves (Kosma et al., 2014). As shown in chapter 3 (Figure 2), MYB41 is highly expressed in roots, a suberin-rich organ. All this data together might suggest that MYB41 is involved in promoting suberin biosynthesis in roots, however it is still highly speculative.

To conclude, MYB41 is a transcription factor involved in the negative regulation of wax biosynthesis in Arabidopsis inflorescence stems. As it was shown in chapter 3 (Figure 6), only the presence of the R2 domain of MYB41 is sufficient to repress the expression of *CER4*/*FAR3*, a main reductase involved in the primary alcohol biosynthesis. Moreover, this transcription factor might be involved in the suberin biosynthesis in Arabidopsis roots as well. However, more research is needed to understand the role of MYB41 in the biosynthesis of this polyester.

4.5 Concluding remarks

The knowledge about wax and TAG biosynthesis is constantly improving. Since the discovery of the TAG pathway 60 years ago and the analysis of first genes involved in wax biosynthesis 40 years ago, the main enzymes taking part in this pathway are characterized. However, the regulation of this pathway, especially upon stress, still stays in the focus of many researchers.

Chapter 2 of this thesis focused on the plants response to wounding. The results included in this chapter led to the conclusion that TAG biosynthesis upon wounding is caused by membrane

damage and remodeling independently from the main wound-signaling hormones - JA-Ile and ABA. Furthermore, two main acyltransferases - DGAT1 and PDAT1 are most likely involved in this process. Accumulating TAG, rich in PUFA, is stored in lipid droplets in and around the wounding site. More research is needed to assess if TAG accumulating upon wounding can be a source of energy for the tissue regeneration. In addition, upon wounding, wax is also synthesized, most likely to seal the damaged area. The wax accumulation upon wounding is dependent on ABA and JA-Ile however it is not clear how the latter one is influencing this process. Moreover, MYB96, one of the main positive regulators of wax biosynthesis, is a key regulator of wax biosynthesis upon wounding, while MYB94, closely related to MYB96, has no impact on this process. In chapter 3, the function of another MYB transcription factor was revealed - MYB41. From data shown in chapter 3, the conclusion can be drawn that MYB41 is a negative regulator of wax biosynthesis. It represses the expression of the main fatty acid reductase - CER4/FAR3- and thus the synthesis of primary alcohols. Besides, this regulation is not stress dependent but rather controlled developmentally. Conclusions considering the wax analysis led to an update of figure 6, where the wound response as well as the function of MYB41 could be added (Figure 10).

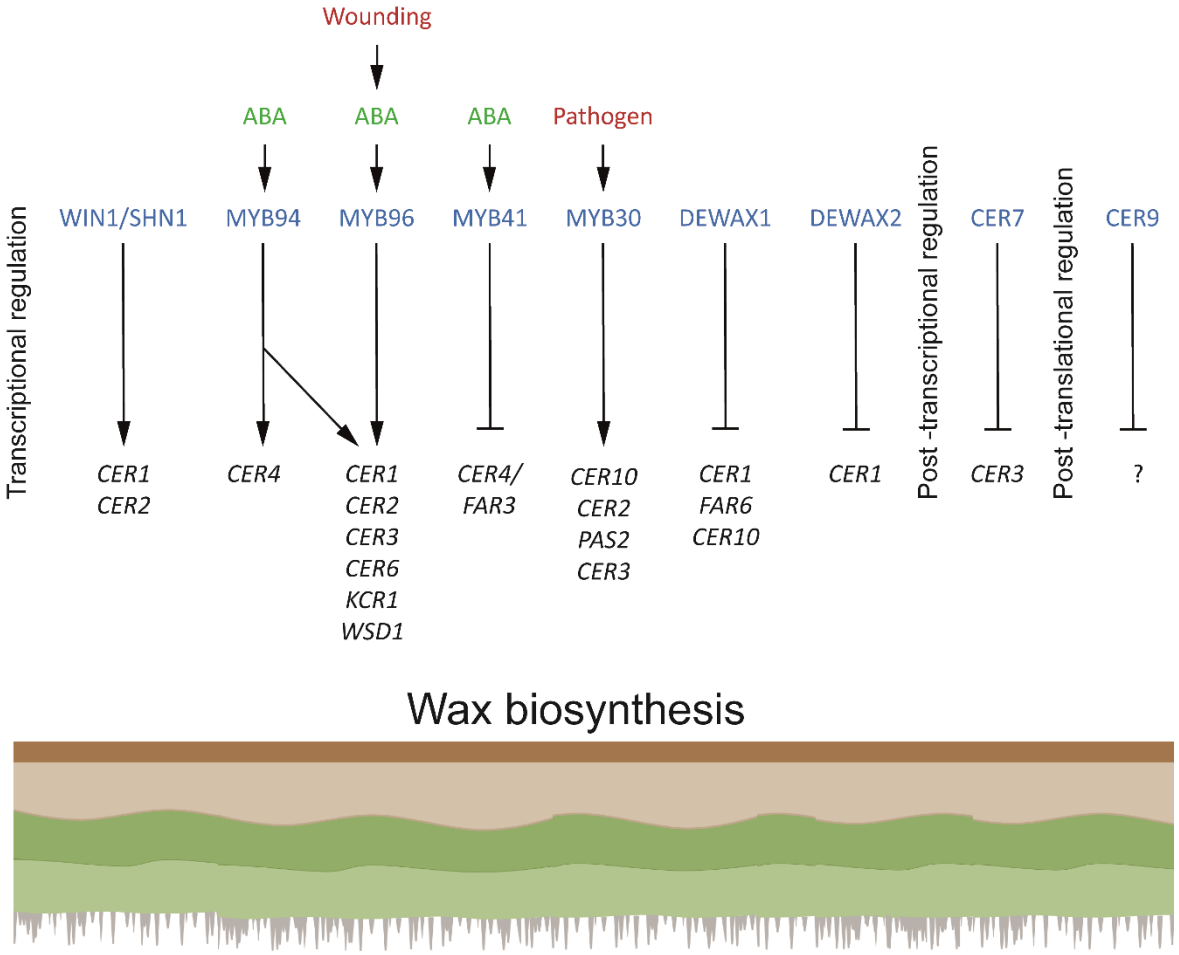


Figure 10. Scheme summarizing the regulation of wax biosynthesis.
Transcription factors regulating positively this pathway are WIN/SHN1, MYB94, MYB96, MYB30 and negatively DEWAX1 and DEWAX2 and MYB41. Moreover, MYB94, MYB96 and MYB30 are regulated by ABA whereas MYB30 is induced upon pathogen attack. MYB96 contributes to wax biosynthesis upon wounding. CER7 is regulating expression of *CER3* post-transcriptionally and CER9 is regulating wax biosynthesis post-translationally.

Chapter 5: Supporting information

Supplemental figure 1 – Expression of *CER6* upon MeJA treatment or mock treatment. The graphic has been taken from the Arabidopsis eFP-Browser, original data from Winter *et al*., 2007. Access 15.04.2019

Chapter 6: Literature

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